ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

THE PRODUCTION OF GENOTOXIC AGENTS FROM AZO DYES

By

Elizabeth Anne Sweeney BSc(Hons.)

A thesis submitted to the Nottingham Trent University of Nottingham in partial fulfilment for the award of the degree of Doctor of Philosophy

The Department of Life Sciences
The Nottingham Trent University
Erasmus Darwin Building
Clifton Lane
Nottingham NG11 8NS

October 1995

To my parents

ACKNOWLEDGEMENTS

I would like to thank Dr. Stephen J. Forsythe for his advice and assistance as the supervisor of this work. My thanks also go to Dr. J. Kevin Chipman (School of Biochemistry, The University of Birmingham), Dr. George Roberts and Dr. Ian G. Coutts (the Department of Chemistry and Physics, The Nottingham Trent University) for their invaluable help, enthusiasm and interest during the course of this study. In addition I am grateful for financial support from The Nottingham Trent University.

I would like to thank the technical staff of the Microbiology Section, Department of Life Sciences for their constant help and encouragement.

Many thanks are extended to my numerous fellow research colleagues past and present. I am also extremely grateful for all those at Glaxo Wellcome for their constant encouragement and support. I could not have done it without them.

And last but not least, to Rob and my family who have stayed with me through it all. Thanks!

ABSTRACT

Azo dyes are the most common synthetic colourings used in the food, pharmaceutical and cosmetic industry. Also known as coal tar dyes, they contain an aromatic ring linked by an azo bond to a second naphthalene or benzene ring. Although the potential carcinogenicity of food colourings has been assessed (largely by mutagenicity testing), and suspected dyes removed from the approved list, the potential hazard from artificial food colourings has not been fully investigated.

The intestinal flora forms a complex ecosystem that metabolises dietary and endogenous nutrients under primarily anaerobic conditions. The ingestion of azo dyes has been proposed as one source of potential genotoxic agents. Many intestinal bacteria are able to reduce the azo bond (termed azo-fission) liberating the substituted amino compounds.

Following bacterial reduction, the genotoxicity of purified amaranth and sunset yellow correlated well with that of an equimolar amount of the aminonaphthol moiety that would result from azo reduction of these dyes. 1-Amino-2-naphthol-3,6-disulphonate and 1-amino-2-naphthol-6-sulphonate (end-products of amaranth and sunset yellow respectively) were highly genotoxic. The other cleavage products in each case, 1-amino-naphthalene-4-sulphonate and sulphanilic acid respectively were non-mutagenic, suggesting that mutagenicity was due solely to the presence of an amino-naphthol compound.

Amaranth and sunset yellow were activated by *Enterococcus faecalis* to a mutagen in *Salmonella typhimurium* TA102 and TA104, but not in *S. typhimurium* TA98 or TA100. Testing the predicted aminonaphthol azo-fission product of both amaranth and sunset yellow resulted in positive mutagenicity. In contrast, the naphthalene moieties were found to be non-mutagenic in *S. typhimurium* TA102 and TA104.

Results showed the production of the active oxygen species, hydrogen peroxide and superoxide radical, from azo dyes reduced by either bacterial or chemical means. Catalase and superoxide dismutase were used to identify the formation of these active oxygens. Further examination of the predicted metabolites from azo fission showed that active oxygen species were only generated for

those compounds with a hydroxyl substituent ortho to the amino function. The protective affects of desferrioxamine and o-phenanthroline against damage by reduced dyes and amino-naphthols indicated the importance of iron in the mechanism behind their toxicity. Protection by antioxidants also indicated the involvement of active oxygen species in the genotoxicity of these compounds.

Oxidative DNA damage, as expressed by 8-hydroxydeoxyguanosine (8-OHdG), was investigated in both calf thymus DNA and *Escherichia coli* DNA. Treatment of DNA with reduced azo dyes and aminonaphthol compounds resulted in an increase in 8-OHdG content. The presence of iron enhanced the formation of 8-OHdG, while catalase resulted in a decrease in 8-OHdG production.

These results suggest that various azo dye products maybe mutagenic, not through N-hydroxylation and esterification which is characteristic of many aromatic amines, but rather through a mechanism involving oxygen radicals and the Fenton reaction.

ABBREVIATIONS

AFB1	Aflatoxin B1
AT	Aminotriazole
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CASE	Computer automated structure evaluation system
CAT	Catalase
CLT	2-amino-5-chloro-4-methyl benzenesulphonic acid
DAB	N,N, Dimethyl-4-aminoazobenzene
DEHP	Diethylhexyphthalate
DETAPAC	Diethylenetriamine
DFO	Desferrioxamine
DMSO	Dimethylsulphoxide
dSOD	Deactivated superoxide dismutase
EBB	Erriochrome blue black
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
FPG	Formamidopyrimidine DNA glycosylase
G1	Glucose
GC-MS	Gas chromatography mass spectroscopy
GSH	Glutathione
HNE	4-hydroxynonenal
HPLC	High performance liquid chromatography
HPLC-EC	High performance liquid chromatography - Electrochemical detection
IDT	Impedance Detection time
LPS	Lipopolysaccharide
MAB	3-methoxy-4-aminoazobenzene
NTA	Nitrilotriacetate
8-OHdG	8-Hydroxydeoxyguanosine
8-OHGua	8-Hydroxyguanosine
PUFA	Polyunsaturated fatty acids
RABIT	Rapid Automated Bacterial Impedance Technique
RF	Riboflavin
ROS	Reactive oxygen species
SOD	Superoxide dismutase
STT	Short term tests
TLC	Thin layer chromatography
UDPGU	UDP-glucuronic acid
UHQ	Ultra high quality

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

INTRODUCTION

The overall aim of this study was to produce a major investigation into the consequences of azo dye reduction by intestinal bacteria. The study was divided into three main parts :

i) the study of the enzymology of azo dye reduction,

ii) identification of the genotoxic/mutagenic metabolite(s) of azo-fission and

iii) determination of the mechanism behind the DNA-damaging affect of the end-products from azo dye reduction.

1.1 AZO COMPOUNDS

Azo compounds are the most common synthetic colourings used in the food, textile, pharmaceutical and cosmetic industries. Also known as coal tar dyes, they contain an aromatic ring linked by an azo bond to a second naphthalene or benzene ring.

Manufacturers have outlined three reasons in favour of using azo dyes:

i) they help intensify the natural colours of products which may be considered too weak by consumers and manufacturers,

ii) azo dyes help overcome variations in colour intensity in the raw materials,

iii) they help replace colour lost during processing and storage due to the action of heat, bleaching and light.

The colour inherent in azo linkage derives from the conjugated system which facilitates pi-electron delocalisation, giving rise to energy absorption at selective wavelengths in the visible spectrum. This permits the synthesis of a vast number of azo dyes of great intensity and variety of colour.

Commercial dyes are prepared mainly by diazotising aromatic primary amines and coupling the diazonium salts with phenols or aromatic amines with free ortho and, or para positions having high electron densities or with other compounds having reactive positions. Unfortunately some intermediates in azo dye manufacture, such as 2-naphthylamine and other aromatic amines, are

carcinogenic or otherwise toxic. Efforts have been made to devise alternative routes of synthesis utilising safer compounds and methods of production (Clarke, 1984).

It appears that metabolic activation is required for all forms of toxicity by azo dyes. Several studies have provided unequivocal evidence that azoreductases from the intestinal microflora, and to a lesser extent from mammals, catalyse cleavage of the azo bond to produce aromatic amines. Aromatic amines induce urinary bladder cancer in man and tumours in some experimental animals (Combes and Haveland-Smith, 1982; Chung, 1983). Examples of representative azo dyes are found in Figure 1.1.

1.1.1 Chemistry of azo dyes

Simple aromatic azo compounds, such as azobenzene, exist preferentially in the trans configuration, which is planar and considerably more stable than the corresponding hydrazo form (Venkataram, 1952). As the complexity of the molecule increases, steric factors maintain the molecule exclusively in the trans form. With simple amino substituted azobenzenes, the basicity of the azo nitrogen increases through delocalisation of the unpaired electrons of the aromatic amine (Figure 1.2).

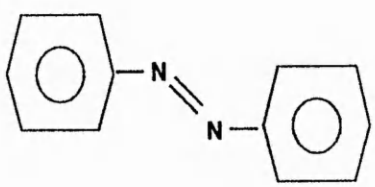
Formation of an azoxy group at the azo bond confers stability on the molecule due to different electro-negativities of the two nitrogen atoms, leading to a somewhat ionic character of the linkage. Dual azoxy groups lead to repulsion of the two positively charged nitrogen atoms, although bond strength remains high. Reduction fission of the azo group, aside from the enzymic processes, can be effected by a number of reducing agents including sodium sulphide, stannous chloride and sodium hydrosulphite. Loss of colour follows reduction and these methods are used in dye detection and destruction when required in dyeing and printing (Stead, 1970).

Quantitative and qualitative characteristics of azo dyes are dependent on:

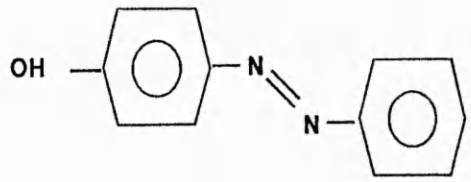
i) number and position of azo groups,

ii) nature of the aromatic nucleus,

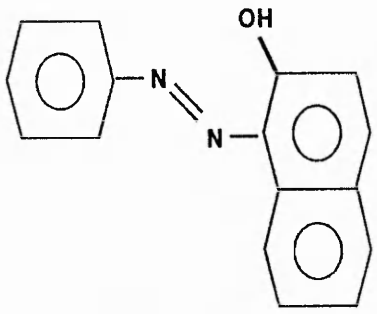
iii) nature and position of substituents, ie hydroxy group, sulphonate group.



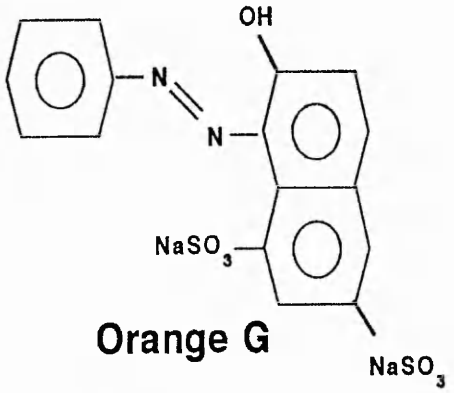
Azobenzene



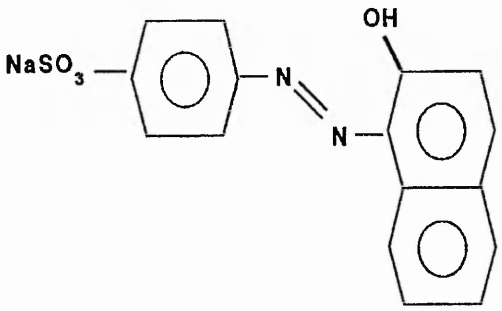
p-Phenylazophenol



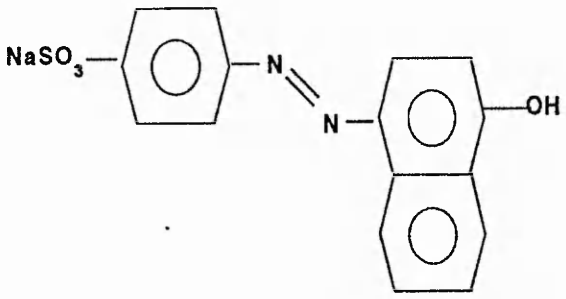
Sudan I



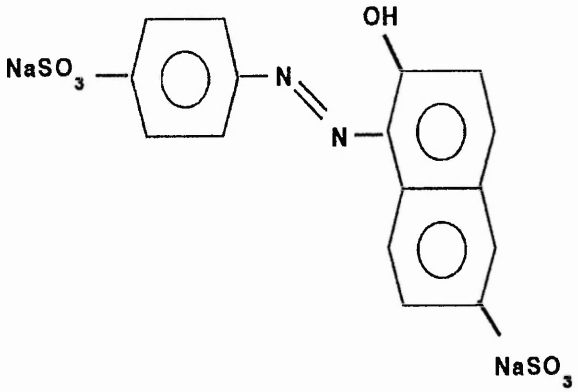
Orange G



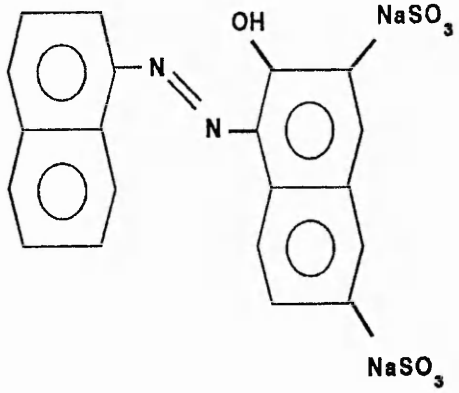
Orange II



Orange I

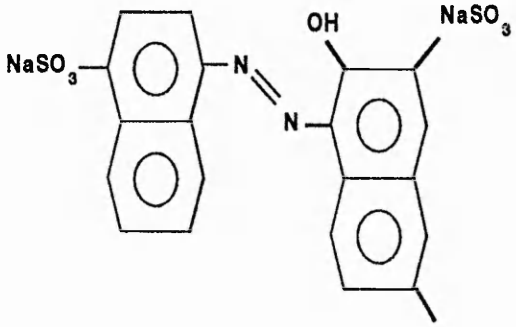


Sunset Yellow

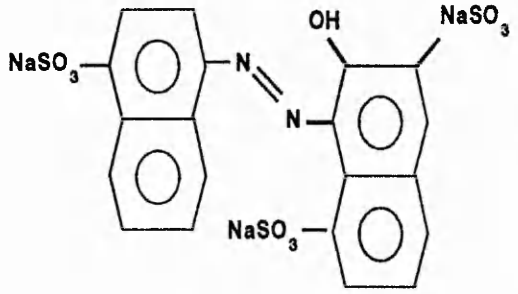


Bordeau Red

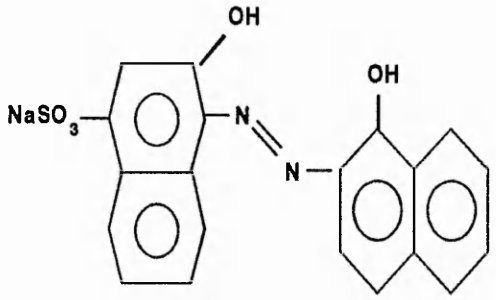
Figure 1.1 Chemical structures of azo dyes



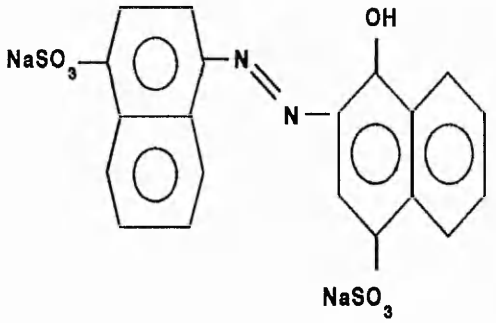
Amaranth



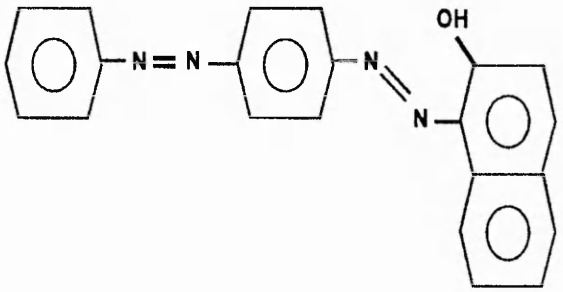
Ponceau 6R



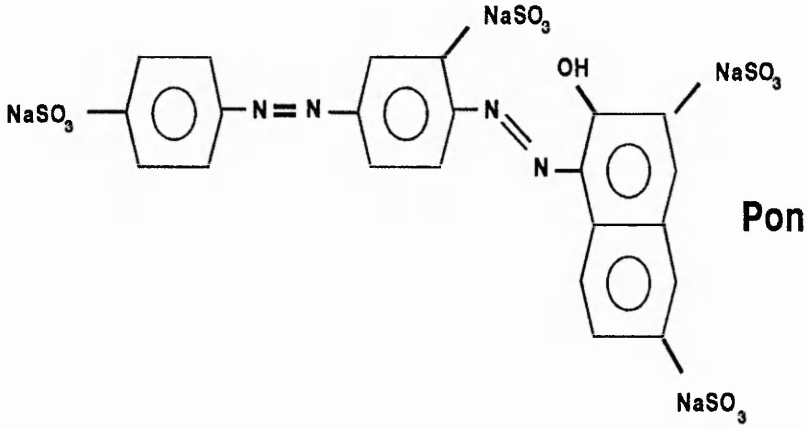
Eriochrome Blue Black B



Carmoisine



Sudan III



Ponceau S

Figure 1.1 Chemical structures of azo dyes (Continued)

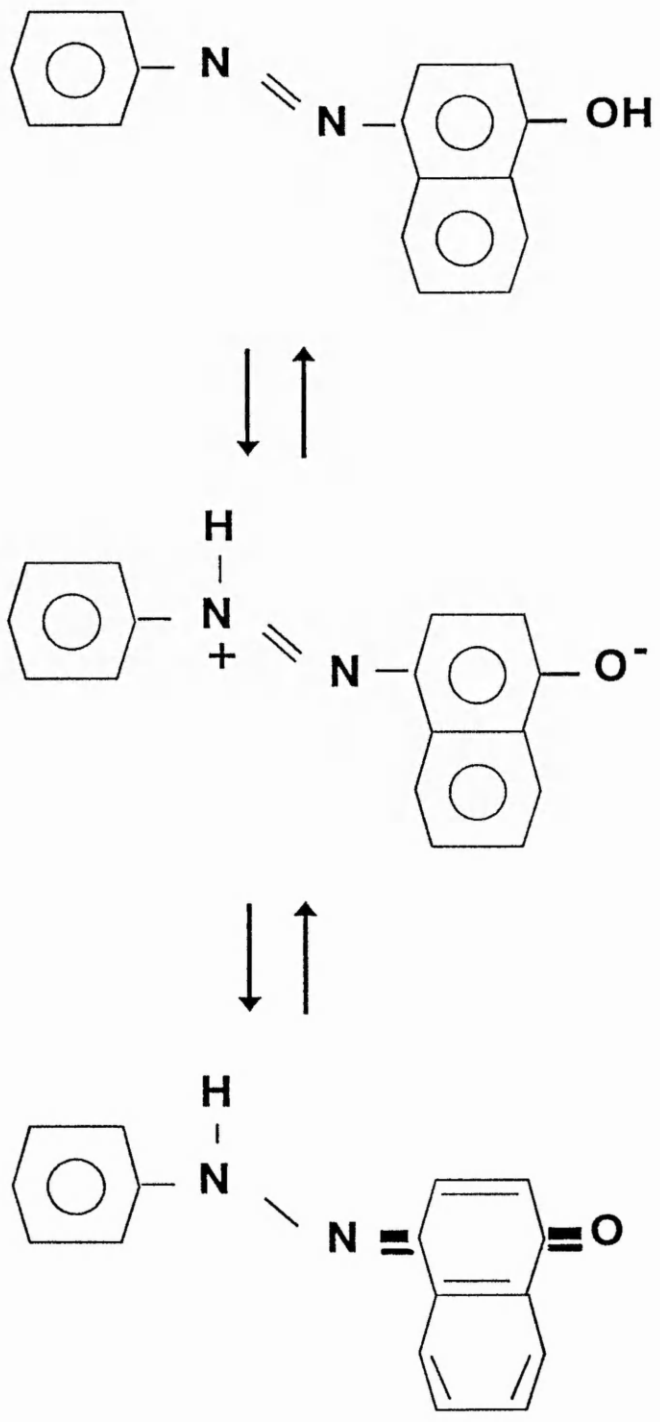


Figure 1.2 Tautomeric equilibrium of 1-phenyl-azo-4-naphthol

1.1.2 Structures and properties of azo dyes

Azo dyes can be separated into lipid soluble and water soluble types. The triphenylmethane colours, which include FD&C blue No 1 (brilliant blue FCF), contain sulphonic acid groups and are therefore water soluble. They are also poorly absorbed from the gastrointestinal tract, undoubtedly due to their low pKs. These colours, because of their strongly acidic nature are very poorly absorbed after oral administration and are largely excreted unchanged in the faeces.

Sulphonated naphthalene azo dyes are water soluble mono-, di- and trisulphonated colours containing a naphthalene ring and an azo linkage to either a second naphthalene or benzene ring. Most of the common food colourings, such as sunset yellow, amaranth and carmoisine belong to this group. The toxicity of these colours may be due to their metabolic products. The less toxic members of this series are sulphonated on both aromatic rings adjacent to the azo group. They would therefore yield only sulphonated fission products which are poorly absorbed (Levine 1991).

The oil-soluble azo dyes as a group are the most toxic. Most contain a naphthalene residue on one side and a benzene residue on the other side of the azo linkage. They are reduced by the intestinal flora of rats, rabbits and dogs, although at a lower rate than the water soluble dyes (Radomski, 1961).

1.1.3 Oxidative metabolism

Most commercially used azo dyes are substituted with large, highly charged groups precluding significant oral absorption of the intact dye and subsequent oxidative metabolism. Studies of sunset yellow and sudan 1 indicate that only a few percent of an oral dose is excreted in the urine with the azo linkage intact (Scheline and Longberg, 1965), apparently metabolism other than reduction does not occur. When intestinal bacteria, the major source of azoreductase for these dyes, are bypassed through intravenous injection, substantial biliary excretion of intact dye is seen (Ryan and Wright, 1961). The lack of azo reduction products in the bile under these conditions may be attributable to the oxygen sensitivity of hepatic mechanisms for reduction of azo dyes. On the other hand, dyes with greater lipid solubility may undergo significant oxidative metabolism.

Early work on N,N-dimethyl-4-aminoazobenzene (DAB) revealed extensive oxidative and reductive pathways (Mueller and Miller, 1950; Miller and Miller, 1969). The known pathways for carcinogen metabolism and activation generally favour oxidation over reduction. For aminoazo dyes, as for aromatic amines, oxidation of an amino group to form the N-hydroxy derivative is considered to be an essential step in the formation of the proximate mutagen. This oxidation is not sufficient for activation of the compounds. Further metabolism to form the ultimate reactive mutagen is required. As shown in Figure 1.3 this can occur by:

i) esterification of the N-hydroxy group (Kadlubar *et al.*, 1976),
ii) non-enzymic protonation of the N-hydroxy group (Kimura *et al.*, 1979),
iii) oxidation to a free radical (Kimura *et al.*, 1982).

Azo dyes lacking the amino group can undergo biotransformation by oxidative splitting to form corresponding quinones and arenediazonium ions (Figure 1.3). The role of such ions in the expression of carcinogenicity is well established (Stiborova *et al.*, 1988a). The benzenediazonium ion, formed on oxidation of sudan 1, is capable of binding to DNA (Stiborova *et al.*, 1988b).

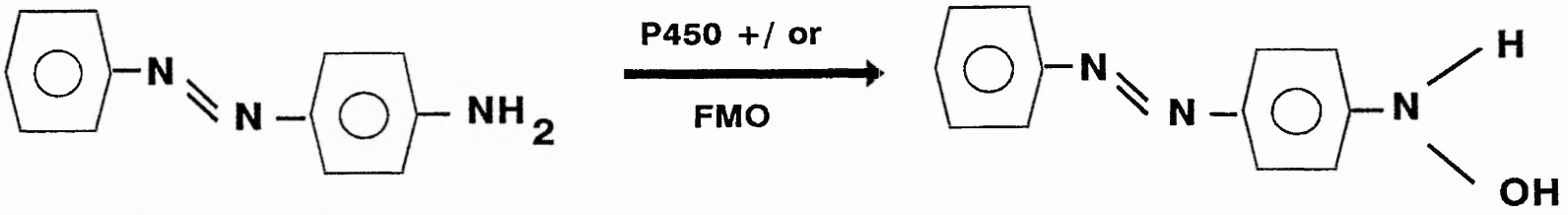
The majority of the above oxidative pathways are catalysed by the microsomal monooxygenase system based on cytochrome P-450 since this comprises a powerful and versatile system for the oxidation of carcinogens.

1.1.4 Reductive metabolism

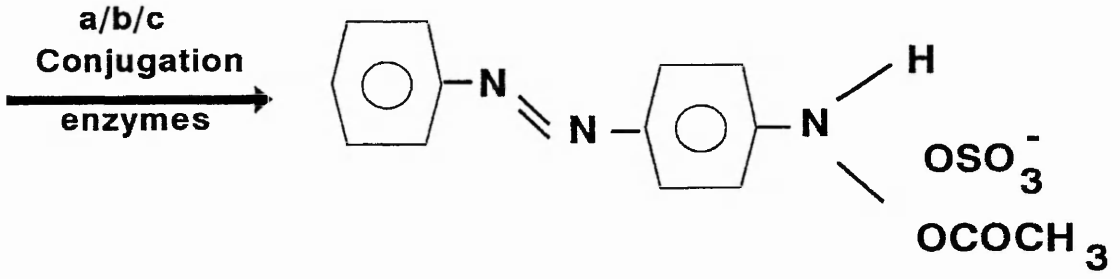
1.1.4.1 Mammalian systems with azoreductase activity

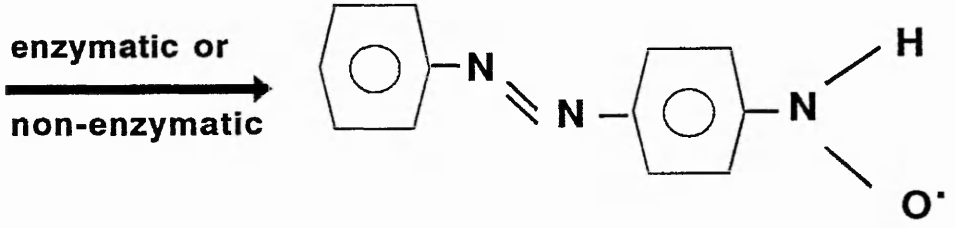
Azo reduction is an important metabolic pathway in the activation and detoxification of azo dyes. Activity is primarily located in the liver microsomes (Fouts *et al.*, 1957). It has been concluded that purified azoreductase is probably identical to purified NADPH-cytochrome P-450 reductase (Hernandez *et al.*, 1967).

Fujita and Peisach (1978) reported that the microsomal reduction of amaranth is almost totally inhibited by carbon monoxide and attributed all of the activity to cytochrome P-450. In microsomal



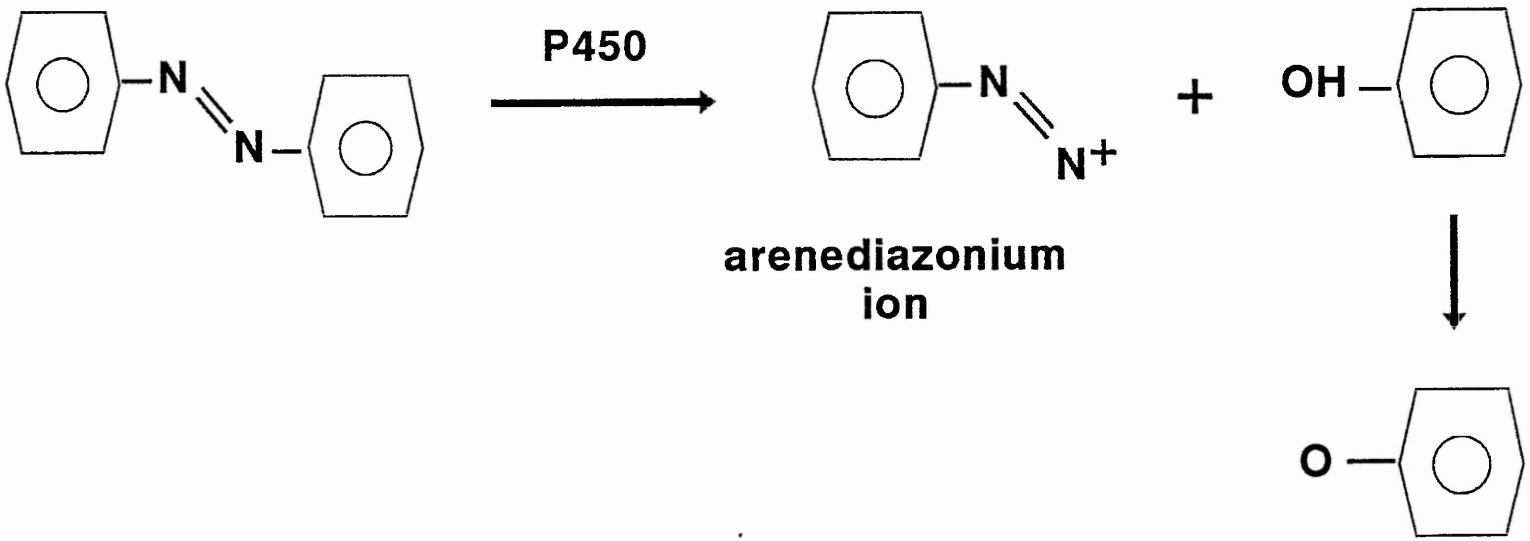
n-Oxidation of aminoazo dyes to form N-hydroxy derivatives





Further metabolism of the N-hydroxy compound

Figure 1.3 Pathways in the metabolism of azo dyes



Oxidation of nonamino azo dyes

Figure 1.3 Pathways in the metabolism of azo dyes (Continued)

systems, activity is proportional to the quantity of cytochrome P-450 present and antibodies to both types of cytochrome inhibit activity (Fujita *et al.*, 1981). Oxygen inhibits and added flavin stimulates reduction (Fujita and Peisach, 1982). The flavin stimulates electron flow directly from flavoprotein (NADPH-cyt-P-450 reductase) to dye. A highly purified form of cytochrome P-450, prepared from phenobarbital-treated rats, also reduces amaranth under anaerobic conditions (Mallett *et al.*, 1985). Here too, added flavin (FMN) stimulates activity although flavoprotein alone does not reduce amaranth. More recent work (Peterson *et al.*, 1988) confirms that cytochrome P-450 is the sole source of oxygen- and carbon monoxide-sensitive amaranth azoreductase in hepatic microsomes. Figure 1.4 shows the proposed mechanism for the microsomal azo reduction of azo dyes.

A structure-activity study of various azobenzenes revealed that a polar electron donating group (amino or hydroxy) para to the azo linkage is required for substrate activity with microsomes. Azobenzene, itself lacking any ring substituents, is unreactive, although its reduction products have been isolated from rat urine after feeding (Elson and Warren, 1944).

Azo reduction is also catalysed by cytosolic enzymes. Unlike the microsomal system, substrate specificity for this enzyme is highly restrictive. Removing or methylating the carboxyl group or shifting it to the opposite ring destroys all activity as does substituting a hydroxyl for the carboxyl, eliminating the possibility that hydrogen bonding from carboxyl to azo nitrogen is critical. The cytosolic azoreductase is insensitive to oxygen and carbon dioxide and is able to use both NADPH and NADH as electron donors.

Although azo reduction has been shown as an inactivation step for DAB and its derivatives (Mueller and Miller, 1950), it seems to be an important pathway for the expression of mutagenicity of benzidine-derived dyes. Rat liver microsomes have shown an inability to reduce azo dyes aerobically (Martin and Kennelly, 1981). Consequently, reducing agents such as riboflavin or flavin mononucleotide are being increasingly used to enhance azo reduction in mutagenicity assays in order to help predict the role of this pathway in the metabolism of azo compounds.

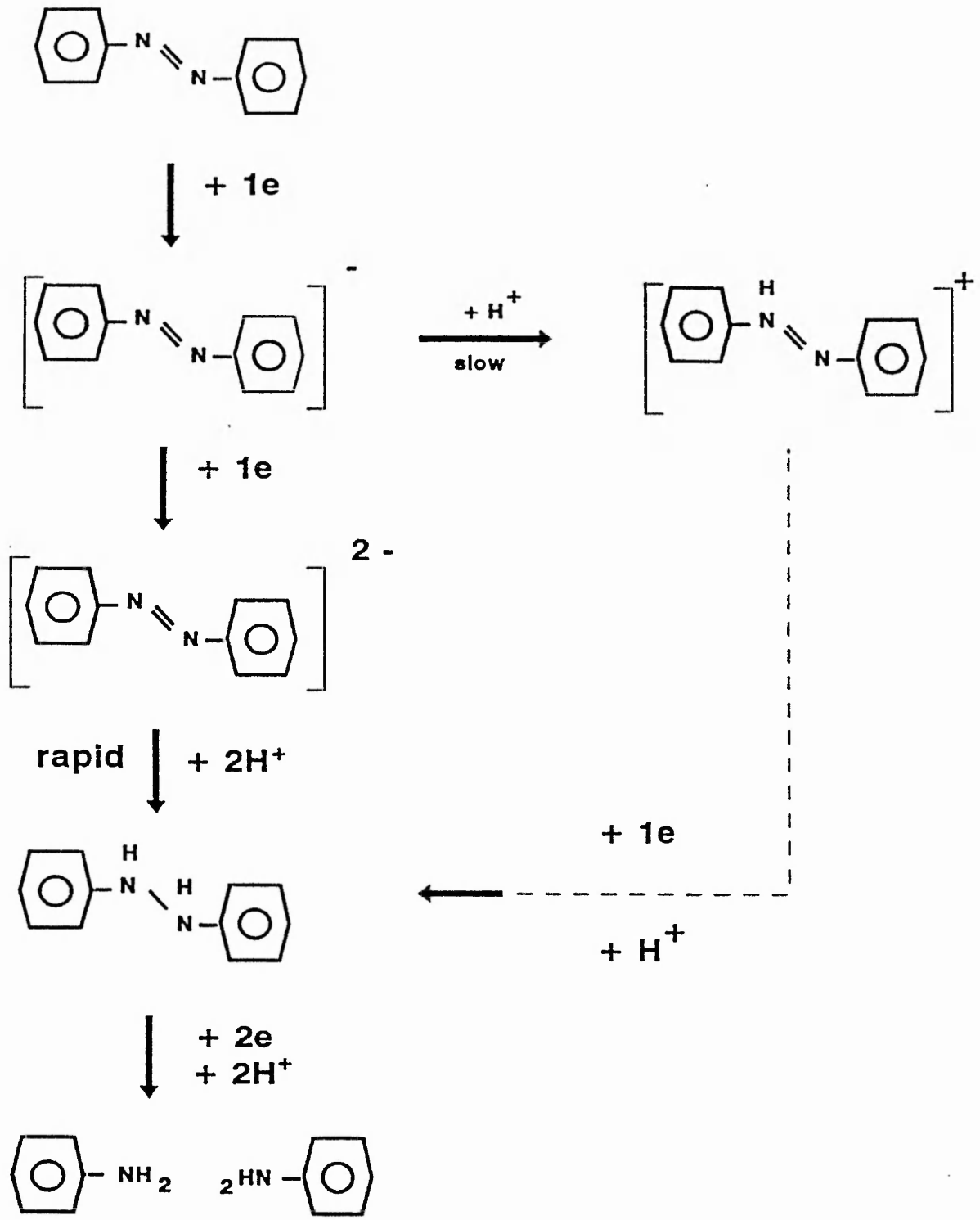


Figure 1.4 Proposed mechanism for the microsomal azo reduction of azo dyes. The first electron is received forming a free radical. Following transfer of the second electron, protonation is rapid forming the true hydrazo intermediate which spontaneously reduces to the primary amines. Protonation of the primary electron reduced free radical also occurs but less readily than protonation of the second electron reduced form.

1.1.4.2 Intestinal microflora with azoreductase activity

The ability of micro-organisms to reduce azo dyes was established long before intestinal bacteria were implicated in the reduction of orally ingested dyes (Walker, 1970). It appears that reduction of the majority of azo dyes is sensitive to oxygen, diminishing the likelihood of hepatic reduction of these compounds. The reduction products of azo dyes have been isolated from urine, focusing interest on the intestinal bacteria (Scheline and Longberg, 1965). Isolation of bacteria from intestinal contents and faeces showed a number of specific species were active in reduction of azo dyes. The reaction appeared to be relatively non-specific (Rowland, 1988; Gingell and Walker, 1971; Dubin and Wright, 1975).

Reduction of certain azo dyes by *Enterococcus faecalis* is stimulated by the addition of FMN or FAD, with NADH and NADP acting as electron donors (Gingell and Walker, 1971). It has been suggested that flavin acts as an electron shuttle between the bacterial enzyme and the dye. The mechanism of action of reduction of the azo linkage involves the formation of a two electron reduction product, ie corresponding hydrazine (pathway 1.1). This hydrazo intermediate is subsequently reduced by a further two electron transfer from NADPH to the primary amine (pathway 1.2).

$$FMNH_2 + R\text{-}N{=}N\text{-}R' \longrightarrow FMN + R\text{-}NH\text{-}NH\text{-}R' \qquad \textit{Pathway 1.1}$$

$$FMNH_2 + R\text{-}NH\text{-}NH\text{-}R' \longrightarrow FMN + R\text{-}NH_2 + R'\text{-}NH_2 \qquad \textit{Pathway 1.2}$$

Inhibition of azoreductases may be caused by the reoxidation of the hydrazo intermediate. This is possible since hydrazo compounds are known to be sensitive to oxidation by air (Hernandez *et al.*, 1967). The oxidation of the hydrazo intermediate can also cause a depletion of NADPH and thus may result in poor yield of the primary amine. In view of their charged nature, it is uncertain whether FAD and FMN could readily shuttle across bacterial cell walls and membranes under physiological conditions. In addition, it is highly unlikely that highly charged sulphonated dyes could pass through the cell wall of these micro-organisms. An electron shuttle could affect the reduction with the dye remaining extracellular. This may also serve as a partial explanation for the

oxygen sensitivity of these reduction reactions, the reduced flavin having a greater affinity for oxygen than for an azo dye. Other electron carriers, such as methyl viologen, neutral red and crystal violet, can all replace FMN, further suggesting that an extracellular shuttle is required for azo reduction.

Larsen *et al.* (1976) found that reduction rates of sulphonated water-soluble azo dyes in caecal preparations from rats depended on the number rather than the position of sulphonated groups in the molecule. Since enzymic reduction of azo dyes may be either a one electron or two electron transfer, the rate of dye reduction is likely to be influenced by the electron density of the azo bond. Other factors which may influence rate of azo reduction include:

i) the nature of the reducing agent,
ii) molecular parameters that influence the ease with which electrons can be accepted by the azo group,
iii) permeability of the bacterial cell wall.

Preliminary comparisons by Wuhrmann *et al.* (1980) on the reduction rates of azo compounds with intact cells and cell extracts strongly indicated that permeation through the cell envelope was a rate limiting factor for the microbial reduction of azo dyes.

Transport of the dye from the external medium through the cell wall to the plasma membrane may be governed by an absorption-desorption equilibrium of the dye at the cell wall material. Wuhrmann *et al.* (1980) indicated that membrane transport had a pertinent effect on the reduction rates of the same dye by various systems, and the reduction of some dyes by cell extracts and not by whole cells.

A concern in the spectrophotometric measurement of dye reduction by bacteria is non-specific absorption to cell walls. Dye absorbance decreases and may vary from one dye to another (Horitsu *et al.*, 1977). The presence of absorbed dyes may affect the ability of the bacteria to reduce other dyes by blocking access to intracellular enzymes.

1.1.4.3 Identification of bacterial azoreductases

Several anaerobic bacteria from human intestinal microflora produce different quantities of extracellular oxygen-sensitive azoreductases (Rafii *et al.*, 1990). The predominant azoreductase-producing bacteria from human intestinal microflora are in the taxonomically unrelated genera *Clostridium* and *Eubacterium*. Rafii *et al.* (1992) showed that part of the structure of azoreductases was conserved in taxonomically unrelated genera of anaerobic bacteria. They concluded that the similar domains in these enzymes which may be involved in the catalytic centre, had some amino acid sequence in common. Similarly conserved regions in the catalytic centres have been shown for other enzymes (Mock *et al.*, 1991 and Moriishi *et al.*, 1991). The azoreductases from two strains of *Pseudomonas* (KF44 and KF46) do not exhibit immunological cross-reactivity with each other (Zimmermann *et al.*, 1982). This was attributed to divergence in the amino acid sequences of the catalytic sites during evolution. These strains, unlike the *Clostridium* and *Eubacterium* of Rafii *et al.* (1992) had different substrate specificities.

Ten strains of bacteria were isolated, on the basis of azo dye reduction, from a semicontinuous culture system containing human intestinal microflora (Rafii *et al.*, 1990). Gel electrophoresis suggested the presence of only one azoreductase isozyme in each bacterium, unlike liver microsomes which contain several different types of azoreductases. At least three types of azoreductase isozyme were isolated from the different bacteria, each isozyme demonstrated variations in size and/or charge. Expression of the isozyme types was determined by using nondenaturing polyacrylamide gel electrophoresis. This showed that each bacterium only produced one type of azoreductase isozyme. All the azoreductases were produced constitutively and released extracellularly and were oxygen sensitive. In all anaerobic bacteria tested by Raffii *et al.* (1990), results supported the involvement of a single protein with both dehydrogenase and azoreductase activities. The presence of two dehydrogenase enzymes in *Cl. clostridioforme*, probably indicates that some anaerobic dehydrogenases have azoreductase activity but that all azoreductases from anaerobic bacteria have dehydrogenase activity.

The azoreductase of *Cl. perfringens* was shown to be a dimer with a molecular weight of 181 kDa (Rafii and Cerniglia, 1990). This is different from that of the enzyme isolated from *Pseudomonas*

KF44 with a molecular weight of 21 kDa and an absolute requirement for a hydroxy group in the 4'-position of the naphthol ring of substrate molecules (Zimmermann *et al.* 1982) The purified enzyme of *Pseudomonas* KF46 is a monomer with a molecular weight of 30 kDa and a requirement of substrates with a 2-naphthol moiety. A survey of the efficiency of various orange dyes as substrates for *Pseudomonas* sp. azoreductase, by the same author, showed that:

i) a hydroxy group in the naphthalene ring was required,
ii) charged groups in proximity to the azo group hinder the reaction,
iii) a second polar substituent on the dye molecule impedes the reaction,
iv) electron withdrawing groups on the phenyl ring accelerate the reaction.

1.1.4.4 Dietary factors affecting azoreductase activity

Many factors affect the activity of azoreductase in gastrointestinal tracts. The addition of bile salts to a washed cell suspension of *Proteus vulgaris* increased the rate of azo reduction about five fold (Allen and Roxon, 1974). Whether this was due to the bile salts causing an increase in permeability of the cell envelope or acting as a chemical 'wetting agent' is uncertain.

Wise *et al.* (1983) demonstrated that the azoreductase activity of faecal flora from rats increased significantly with increasing protein in the diet of the rat. The shift from a grain to a meat diet also caused an increase in azoreductase activity in the rats' faecal flora (Goldin and Gorbach, 1976). Total activity of microbial azoreductase per caecum also increased when rats were fed a fibre-free diet (Rowland *et al.*, 1983).

Glucose has been shown to stimulate the reduction of tartrazine by *Proteus vulgaris* (Roxon *et al.*, 1966), but inhibits reduction by *Bacteroides fragilis* (Chung *et al.*, 1978). It has been proposed that a deficiency of dietary fibre may be one of the causes of certain colonic disorders including cancer of the colon and rectum. When the ingested fibre reaches the colon, it will be partially digested and will release soluble sugars like glucose. Glucose will inhibit azoreductase activity, thus decreasing the new production of aromatic amines which are potential carcinogens in the gastrointestinal tract (Burkitt, 1976).

1.1.4.5 Algae and fungi with azoreductase activity

The reduction of azo dyes by algae is dependent on the molecular structure of the dye, species of algae used and environmental conditions. Azo compounds with an amino or hydroxy group are more likely to be reliably degraded than those with a methyl, methoxy, sulpho or nitro group. Amino or hydroxy groups have been shown to act against the inhibition of sulpho groups on azo reduction by *Chlorella vulgaris* (Jinqi and Houtian, 1992). This study also showed that degradation of sulphonated dyes was poor. Degradation of azo dyes by algae follows a similar pathway to bacterial degradation. The azo bond is cleaved to produce aromatic amines. These can be completely degraded to carbon dioxide or to other intermediates (Kulla, 1983). This area needs further study to ascertain the end-products.

Some white rot fungi also decolourise and degrade various synthetic dyes, including azo and anthraquinone dyes, under aerobic conditions (Zhou and Zimmermann, 1993). The white rot fungus *Phanerochaete chrysosporium* was reported to aerobically degrade the azo dyes orange II, tropaeolin O and congo red (Zhou and Zimmermann, 1993). Much of the dye was adsorbed by the mycelia of the fungus, but considerable degradation was also shown. In a recent study by Spadaro *et al.* (1992), *P. chrysosporium* was shown to be able to mineralise the ^{14}C-ring-labelled azo dyes 4-phenylazophenol, 4-phenylazo-2-methoxyphenol, disperse yellow 3, disperse orange 3 and solvent yellow under nitrogen limiting and ligninolytic conditions. Much work in this area is required before the detailed mechanism of azo reduction by these organisms is understood.

1.1.5 Toxicological significance of azo reduction

It is likely that the toxic or carcinogenic effects of azo dyes are due to reductive cleavage products. This suggests that tests on intact molecules are not indicative of all the potential hazards, since the reduction cleavage molecules and not the intact molecule may be genotoxic. Both orange II and lithol red can be converted into 1-amino-2-naphthol, which has been reported to be carcinogenic in mice (Bonser *et al.*, 1956; Chung, 1983). This suggests that the toxicity of an azo dye depends to a large extent on the rate at which bacterial systems reduce the azo linkage.

Although hepatic enzymes can metabolise a variety of azo compounds, the highly sulphonated intact azo dyes are poorly absorbed from the intestine after oral administration. However, reductive cleavage products formed *in situ* by the intestinal microflora are rapidly absorbed and metabolised by the liver and excreted in the urine and bile (Honohan *et al.*, 1977).

It is clear that the intestinal microflora play an important role in the metabolism of azo dyes. Azo reduction is by far the most important reaction in relation to the toxicity and mutagenicity of these compounds.

1.1.5.1 Carcinogenesis

Numerous azo dyes have been tested for carcinogenicity. The liver is the major site of tumorigenesis for most dyes. Carcinogenicity has been confirmed by epidemiological studies, which indicate that workers in the dye industry have an abnormally high incidence of bladder tumours (Haley, 1975; Powell *et al.*, 1979).

Some of the negative carcinogenic results reported by many groups on azo dyes may be attributable to inadequate dosage, lack of sufficient purity of azo dye preparation, which may be suitable for commercial usage, but not for carcinogenicity testing. Reports of long-term feeding experiments rarely mention purification of dye preparations.

Early observations on the tight binding of dye to rat liver after DAB administration as well as its lack of spontaneous activity suggested a relationship between binding and carcinogenesis and the necessity for metabolic intervention (Miller, 1978). Subsequent recognition of the genetic role of DNA established covalent binding to nucleic acid as a primary event in chemical carcinogenesis. This suggests that DAB may also be activated via N-hydroxylation.

Other routes of activation of azo dyes have been considered. Nitroxide radicals are reported to form *in vitro* from N-hydroxy-2-methoxy-aminoazobenzene and N-hydroxy-aminoazobenzene as well as *in vivo* after feeding rats with 3'-methyl-DAB (Kimura *et al.*, 1979). Incubation of DAB or MAB with rat liver microsomes generates NADPH-dependent EPR-detectable free radicals (Kimura *et al.*, 1979). 3-Methoxy-4-aminoazobenzene (MAB) is somewhat more active in this

respect and the signal is inhibited 40% by carbon monoxide, suggesting the involvement of cytochrome P-450. It was postulated that nitroxide radicals bind to DNA and a correlation was demonstrated between radical formation and carcinogenic activity of a series of substituted MAB derivatives. To what extent such a radical contributes to azo dye carcinogenesis is unknown, although the role of free radicals in chemical carcinogenesis has long been postulated (Marnett, 1987). These include oxygen radicals possibly generated through reactive metabolic intermediates. It has been proposed that both superoxide and hydrogen peroxide are generated from the nitroxide formed from N-hydroxy MAB (Nakayama *et al.*, 1983). In addition, nitroxide radicals of several aromatic amines bind to microsomal membranes and may subsequently induce formation of activated forms of oxygen (Stier *et al.*, 1980). Most of the carcinogenic amines tested by Stier and colleagues (1980) formed nitroxide radicals while most of the noncarcinogens did not.

A novel route of activation was suggested by Stiborova *et al.* (1988a&b), who proposed the formation of benzenediazonium ion from the carcinogen 1-phenyl-2-hydroxy-naphthalene (sudan I) through an oxidative reaction catalysed by rat liver microsomes. This reactive intermediate could then bind covalently to DNA.

Ortho hydroxy amines with aromatic systems containing two or more rings are generally found to be carcinogenic when tested by bladder implantation (Stanaka *et al.*, 1980). Derivatives of the ortho hydroxy amines vary in their carcinogenic activity according to the position of the substituent. To be carcinogenic an ortho hydroxy amine must have:

i) an amino group with at least one free hydrogen atom,
ii) either a carbon atom or a carbon-carbon bond with a high electron density, and this region of high electron density will be the point at which interaction with body tissue will take place.

Demonstration of the carcinogenicity activity of the ortho hydroxy amines 2-amino-1-naphthol, 1-amino-2-naphthol and 3-hydroxy-4-amino-diphenyl under the conditions of bladder implantation strongly support the hypothesis that aromatic amines induce cancer by virtue of their transformation in the body to ortho-hydroxy-amines. This activity may be due to:

i) direct reaction of the ortho hydroxy amines with the tissue constituents,
ii) conversion of the ortho-hydroxy amines to the true carcinogens by the enzymes of the tissues, or

iii) conversion of the ortho hydroxy amines to the true carcinogens chemically in the pellet in the urine.

1.1.5.2 Mutagenesis

The widespread acceptance of mutagenesis as a major mechanism for conversion of a normal cell to a tumour cell has led to mutagenicity testing as a short-term, inexpensive means of screening for potential chemical carcinogens. Azo dye carcinogenicity stemming from chronic feeding appears to require an intact azo linkage. In contrast, most mutagenicity studies imply azo reduction is a prerequisite for activity. The *Salmonella typhimurium* test developed by Ames (1976) is the most widely applied assay for mutagenicity, but others include the genotoxicity assays.

A study (Prival *et al.*, 1988) with several food dyes, including amaranth, showed little or no mutagenesis with or without FMN-supplemented S9 activation. However, extracts of aqueous solutions of FD&C red No. 40 were mutagenic after chemical reduction, as were very high concentrations of the dye itself, suggesting that activity was due to lipophilic contaminants. Each parent dye was inhibitory in mutagenesis tests with chemically reduced metabolites. Similar discrepancies in mutagenic results between crude and purified dyes have been reported (Reid *et al.*, 1984). These factors bring into question the validity of much of the mutagenic and carcinogenic testing. Some purified dyes may be less biologically active than previous testing suggests. However, if dyes are utilised mainly in impure form the activity of the entire preparation must be considered.

Mutagenicity of chemically reduced azo dyes is consistent with biological findings. Unfortunately many aromatic amines are too unstable in air to be tested in conventional mutagenicity test systems. This is true of many dyes permitted for human consumption which have hydroxyl substituents ortho to the amino function. For most reduced products S9 activation is required, confirming that the reduced primary amine is not the final activated form (Joachim *et al.*, 1985). Tartrazine, which is widely used in human consumption, yields mutagenic urinary and faecal products after oral feeding. Since this dye is highly sulphonated, and therefore charged within a broad range of physiological pH, it can be assumed that absorption is minimal and that reduction is entirely due to the action of the intestinal microflora (Henschler and Wild, 1985). In structure activity studies, sulphonation and

carboxylation reduce mutagenicity of the amine probably through resistance to metabolic activation (Soderland *et al.*, 1980).

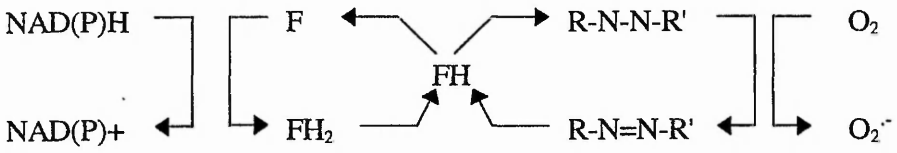
1.1.5.3 Metabolites of azo dye reduction

The reductive cleavage products formed *in situ* by intestinal bacteria are rapidly and extensively absorbed, further modified by the liver and excreted in the bile and urine (Walker, 1970). Sulphonated amines produced by azo reduction, after oral dosing, frequently occur in the urine or bile but not in faeces in free, conjugated or modified forms. Using rabbits it was found that free and acetylated sulphanilic acid was excreted in the urine in amounts equivalent to 70, 77 and 96 per cent of the oral dose of orange II, sunset yellow FCF and tartrazine respectively (Daniel, 1962). 1-Amino-2-naphthol-6-sulphonic acid occurs in the urine at rates of 55 per cent of the oral dose of sunset yellow. The increased absorption compared to the parent azo compound may be due to the effect of basic amino groups. All of the metabolites are more lipophilic so they more readily cross the intestinal mucosa.

Therefore there is considerable uncertainty concerning the mutagenic potential of azo dyes and their derivatives. There is so much splitting of the azo-linkages that it would appear advisable to make special studies of the amine moieties that are formed.

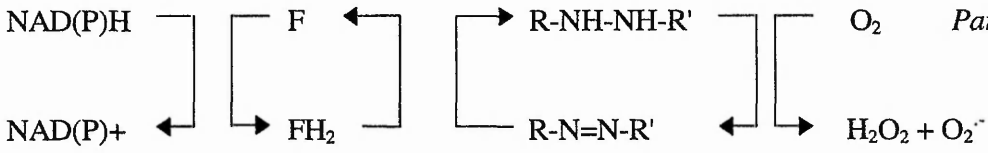
1.1.6 The generation of oxidative mutagens

Studies by Mason *et al.* 1978, showed that a diazonaphthol dye, sulphonazo III was rapidly reduced by hepatic microsomes under nitrogen or carbon monoxide. In addition to the amine cleavage products and the hydrazine intermediate after biological reduction of sulphonazo III, an azo anion radical metabolite was detected by ESR in microsomal incubations under nitrogen or carbon dioxide (Mason *et al.*, 1978). The reaction of this free radical with oxygen appears to mediate the reduction of oxygen to the superoxide free radical (pathway 1.3). This air oxidation of the radical metabolite regenerates the parent azo compound and appears to be responsible for the oxygen inhibition of microsomal azo reduction of sulphonazo III and possibly other oxygen sensitive azoreductases (Gingell and Walker, 1971).



Pathway 1.3

Studies of the azo reduction of neoprontosil (Mason *et al.*, 1978) postulated a similar mechanism (pathway 1.4). They proposed that the first intermediate in the reduction was a second electron reduction product, the corresponding hydrazine derivative. Oxygen inhibition of the reduction resulted from oxidation of the intermediate.



Pathway 1.4

The metabolism of the dye leads to the stimulation of oxygen reduction to superoxide and/or and the oxidation of NADPH.

In addition to the above active oxygen species, there is growing evidence that iron-oxygen complexes may play a key role in several types of oxidative mechanisms. A superoxide anion will form a complex with Fe^{2+} to yield a 'perferryl' radical, $[2Fe^{2+}O_2]'$ (Farr and Kogoma, 1991). This species is not thermodynamically capable of undergoing oxidative reactions with most biomolecules, but will undergo a series of reactions to produce the ferryl radical, $[2Fe^{2+}O]$. This species is electron rich, has radical characteristics and is not spin restricted in its reactions. Consequently it is proposed as one of the major initiating species of lipid peroxidation and possibly DNA damage as well. The formation of the perferryl radical will be enhanced under conditions of increased superoxide anion (Farr and Kogoma, 1991).

A study undertaken by Nakayama and colleagues (1983) detected the generation of hydrogen peroxide and superoxide radical after auto-oxidation of active metabolites of naphthamine and amino azo dyes. The mechanism by which these species are formed is not clear, but it may be specific for ortho-hydroxy aromatic amines and involve the reaction postulated by Nakayama and

colleagues (1983). There may be a requirement for Fe^{2+} when the superoxide anion is converted, via hydrogen peroxide, to the highly reactive hydroxyl radical which is known to damage DNA (Figure 1.5). Such a system may be realised in the microsomal system as well as *in vivo*.

1.2 CHEMICAL CARCINOGENESIS

The one in four incidence of cancer in industrialised countries, has been related largely to exposure to carcinogens in our environment, food and the workplace (Figure 1.6, Lijinsky, 1993). Cancer in humans can appear in a great variety of organs, frequently differing in incidence from one part of the world to another (Parkin *et al.*, 1988).

In many respects chemical carcinogens are similar to drugs and other toxic agents. Carcinogens in a given experimental setting show dose response relationships (Schmahl *et al.*, 1989). Carcinogens interact with other environmental agents. Chemical carcinogens of the type that have the ability to react with DNA differ from most other kinds of toxins in that:

i) their biological effect is persistent, cumulative and delayed,
ii) divided doses are in some cases more effective than an individual dose,
iii) the underlying mechanisms, particularly with respect to interaction and alteration of genetic elements and other macromolecules are distinct.

1.2.1 Prominent discoveries in chemical carcinogenesis

Several types of chemicals were discovered to be carcinogenic in experimental animals after having first been suspected of causing cancer in man (Schmahl *et al.*, 1989). In the late eighteenth century the English physician Percival Pott established a relationship between soot, coal tars and cancer. He observed that many of his patients with cancer of the scrotum were chimney sweeps. Later in the 1920s, Kennaway and colleagues fractionated coal tar and discovered the carcinogenic potency of pure polynuclear aromatic hydrocarbons. including dibenz[a,h]anthracene and benzo[a]pyrene.

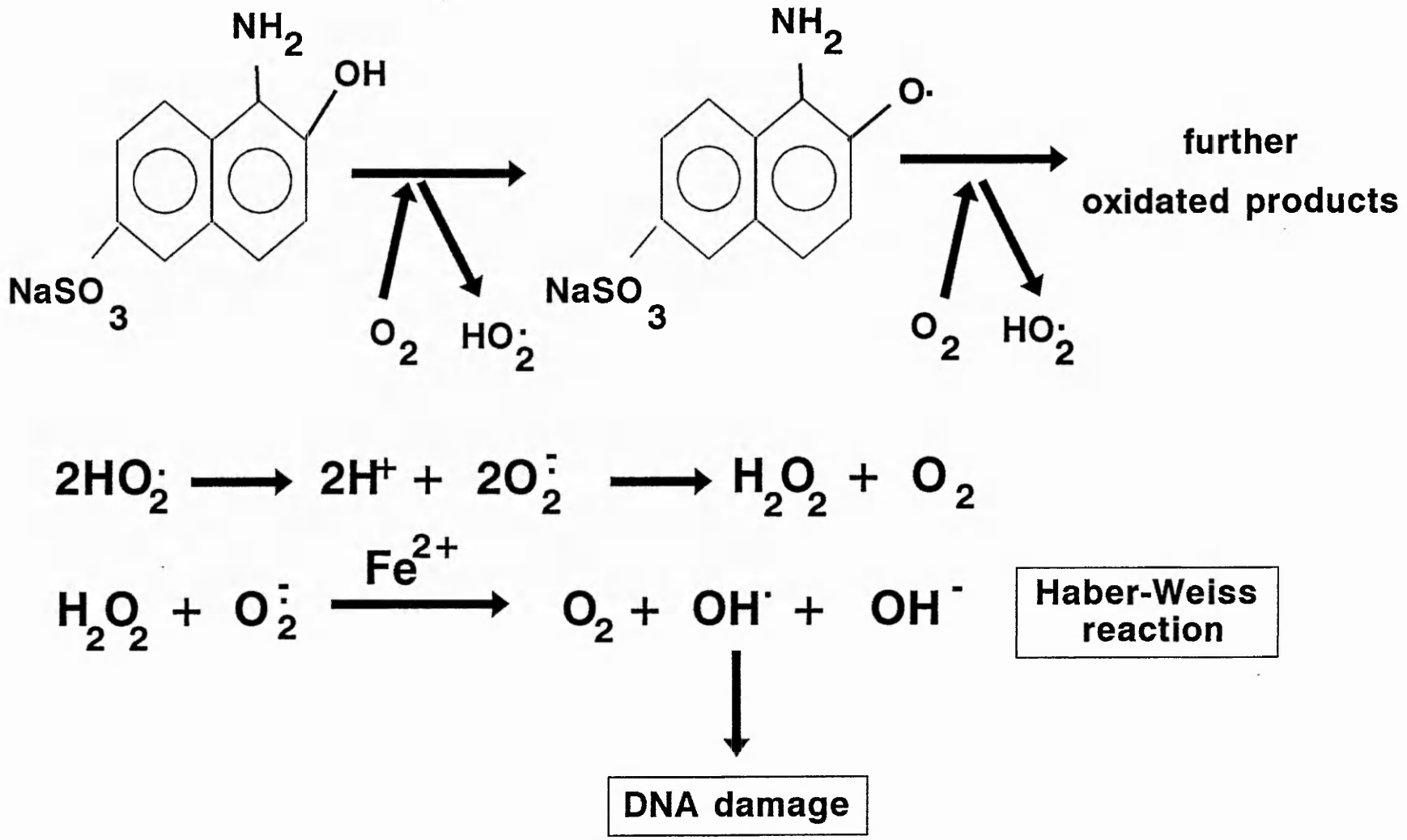


Figure 1.5 Possible mechanism for production of superoxide anions

Adapted from Nakayama *et al.*, 1983

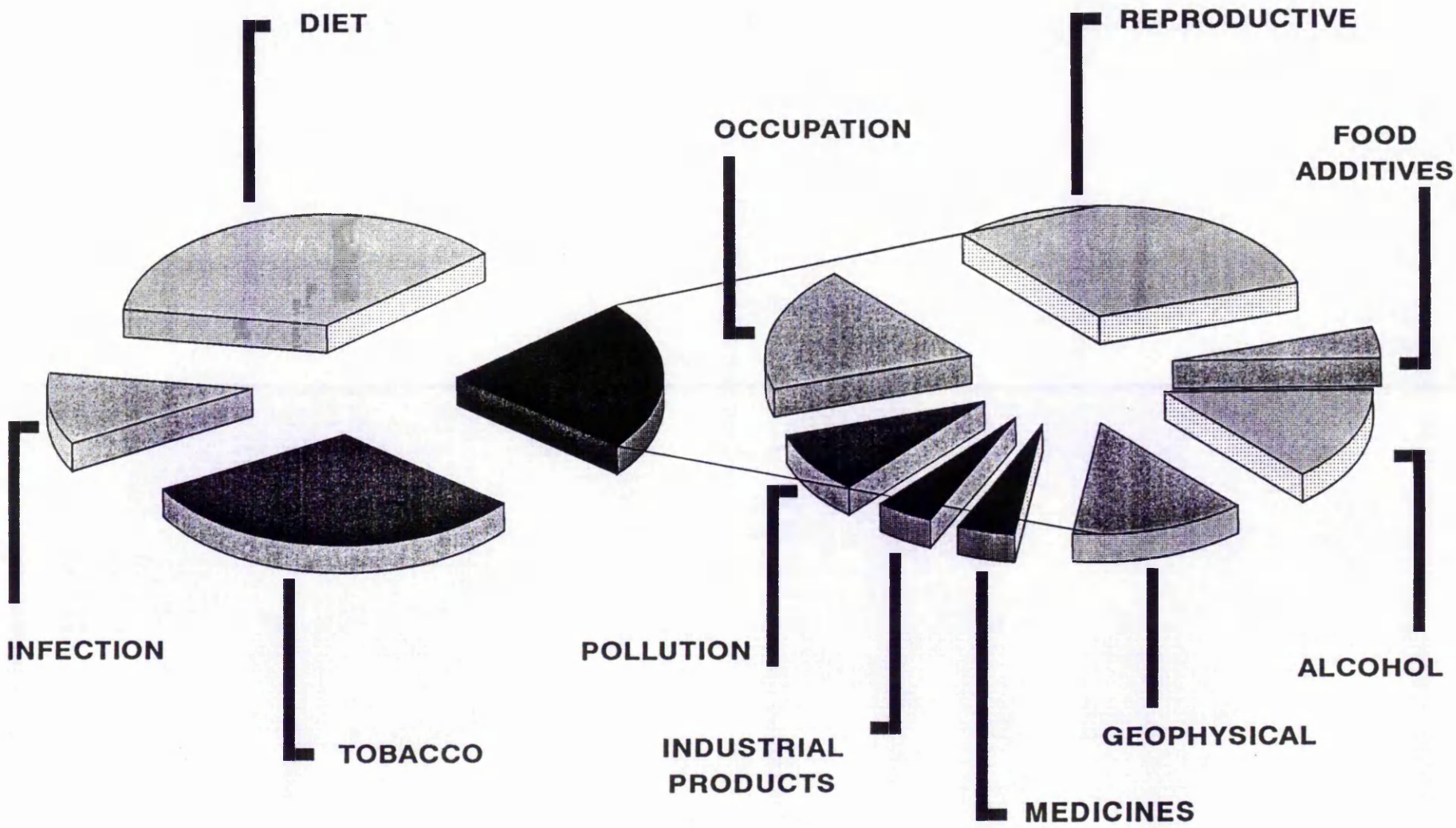


Figure 1.6 Proportions of cancer deaths attributed to various factors
Adapted from Schmahl *et al.*, 1989

In 1895, the German physician, Rehn, noted cancer of the urinary bladder among workers in the dye industry. In 1937, Hueper and colleagues found evidence linking cancers and the amines to which these workers were exposed. Heuper found that 2-naphthylamine caused bladder cancer in dogs, reproducing the lesions seen in humans. Between 1932 and 1934, Kinosita and Yoshida independently discovered some azo dyes caused carcinogenicity in rodents. Hepatocarcinogenicity of *o*-aminoazotoluene (Sasaki and Yoshida, 1935) and N,N-dimethyl-4-aminoazobenzene was demonstrated.

1.2.2 Definition of chemical carcinogenesis

Chemical carcinogens are defined by their ability to induce neoplasms. A neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissue. It is a heritably altered, relatively autonomous growth of tissue (Pitot, 1989). The following responses are accepted as evidence for induction of neoplasms:

i) the presence of types of tumours not seen in the controls,
ii) an increase in the incidence of tumour types occurring in controls,
iii) the development of tumours earlier than in controls,
iv) an increased multiplicity of tumours.

There are many different types of neoplasms. They vary from small, slowly growing nodules which have virtually no affect on the individual (benign), to rapidly enlarging quickly spreading tumours that kill in a short time (malignant) (Pitot, 1989).

1.2.3 Mode of action of chemical carcinogenesis

Carcinogens or their metabolites are believed to act as electrophiles (electron deficient species) which attach to electron rich or nucleophilic sites in nucleic acids, proteins or other cellular macromolecules. Figure 1.7 gives an overview of the critical steps in tumour initiation by chemical carcinogens (Chambers, 1985). Covalent adducts are formed on reaction of chemicals with DNA. These adducts are normally removed by the DNA repair systems and normal DNA is regenerated. If one of these adducts is not repaired a mutation is produced. This can be any change in DNA

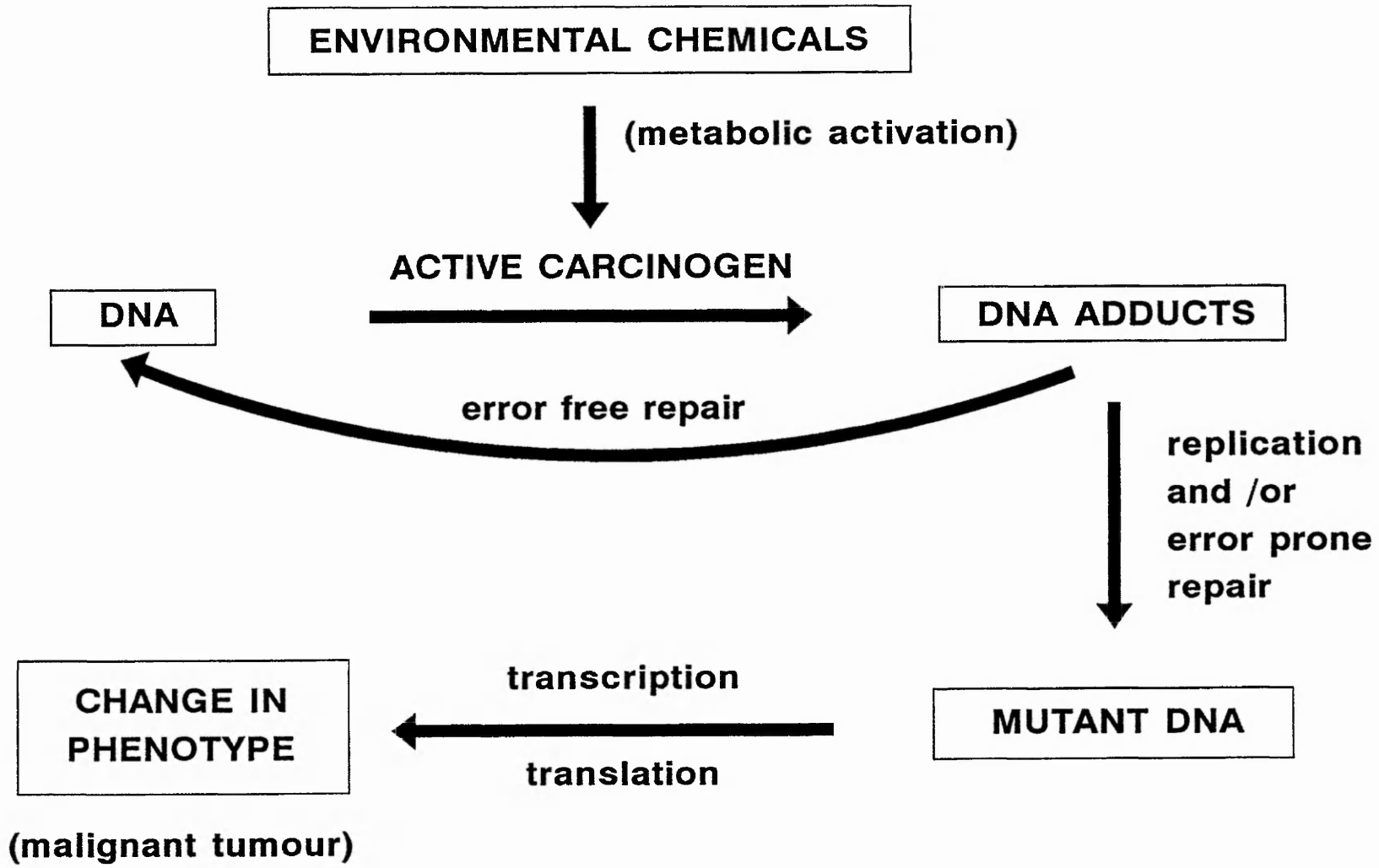


Figure 1.7 Chemical carcinogenesis: A biochemical overview

Adapted from Chambers, 1985

sequence, including base substitution, frameshift, large deletion, insertion or rearrangement. This change is passed on by transcription and translation to produce in some cases a recognisable change in phenotype. This phenotypic change can manifest as a malignant tumour.

The positions in the bases that are attacked by reactive intermediates differ depending on the parent carcinogen. Polycyclic aromatic hydrocarbons usually attack the amino group in guanine and to a lesser extent those in adenine or cytosine (Jeffrey *et al.*, 1976). Arylamidation at the 8-position of guanine has been shown to occur with aromatic amides (Miller and Miller, 1976). Nitrogen mustards, β-propiolactone and dialkylnitrosamine were shown to attack at the 7-position of guanine, although other positions were also attacked. Nitrosoalkylureas formed 7-alkylguanines and have also been shown to attack the O-6 of guanine (Jeffrey *et al.*, 1976).

Chemical carcinogens can be divided into two broad classes, those that act directly and those that require metabolic activation. Direct acting carcinogens include nitrogen or sulphur mustards, methyl methanesulphonate, ethylene imines, bis(chloromethyl)ether or other chemically reactive compounds. Most environmentally important carcinogens including aromatic amines, amino azo dyes and aflatoxins as well as naturally occurring carcinogens require activation.

1.2.3.1 Direct acting carcinogens

These compounds because of their alkylating or acylating capability react with the nucleophilic sites on proteins, nucleic acids or other cellular macromolecules without the necessity for metabolism. Potency of activated compounds may be considerably less than expected due to solubility, steric configuration of molecules involved or reactivity with water or other similar molecules before it reaches the target site.

Direct acting carcinogens include methyl methanosulphate which acts at the 7-position of guanine in DNA or RNA to produce 7-methylguanine. 7-Bromomethylbenz[a]anthracene and its 1,2-methyl analogue are halogen compounds with a polycyclic aromatic nucleus. They form adducts on the amino groups of guanine, adenine or cytosine residues (Weisburger, 1978).

1.2.3.2 Carcinogens requiring activation

Most chemical carcinogens are converted by metabolic activation to electrophiles which in turn react with nucleophiles in the cell. The metabolic pathways and activated molecules depend on the parent compound.

An important activation system works in polycyclic aromatic hydrocarbons. After oxidation the parent compounds become potent carcinogens. The activation system involves a monooxygenase system that utilises molecular oxygen, NADPH and cytochrome P-450. Oxidation of benzo[a]pyrene produces benzo[a]pyrene diolepoxide which reacts with DNA. Epoxide hydrase catalyses the hydrolysis of the 7,8-epoxide but not the 9,10-epoxide, thus the reductive metabolite is accumulated rather than deactivated to tetraol. Cytochrome P-450 is an inducible protein and is turned on by polycyclic hydrocarbons. The epoxide pathway is not unique to hydrocarbons. Other carcinogens such as aflatoxin B1 and some halogenated olefins are activated through this route.

Metabolic products are also responsible for carcinogenicity of aromatic amines and aminoazo dyes. This is substantiated by the fact that large quantities are required to elicit a response and that no site of local action could be detected.

1.2.3.3 Repair systems

Regardless of the exact mechanism, active carcinogens react with DNA to form stable covalent adducts. In most cases these adducts are removed by one of the DNA repair systems. There are two basic types of repair:

i) Adaptive repair

ii) Excision repair.

Adaptive repair removes simple alkyl adducts (methyl, ethyl) directly, regenerating DNA (Figure 1.8). The protein alkyl transferase, inducible in bacteria, attacks the adduct stoichiometrically (Lindahl, 1982). This repair is biochemically error free. The reaction occurs at the alkyl group without breaking any DNA bonds. The purine or pyrimidine residue that is regenerated by this type of repair is always the same as the parent. Thus mutations cannot be produced by alkyl repair of

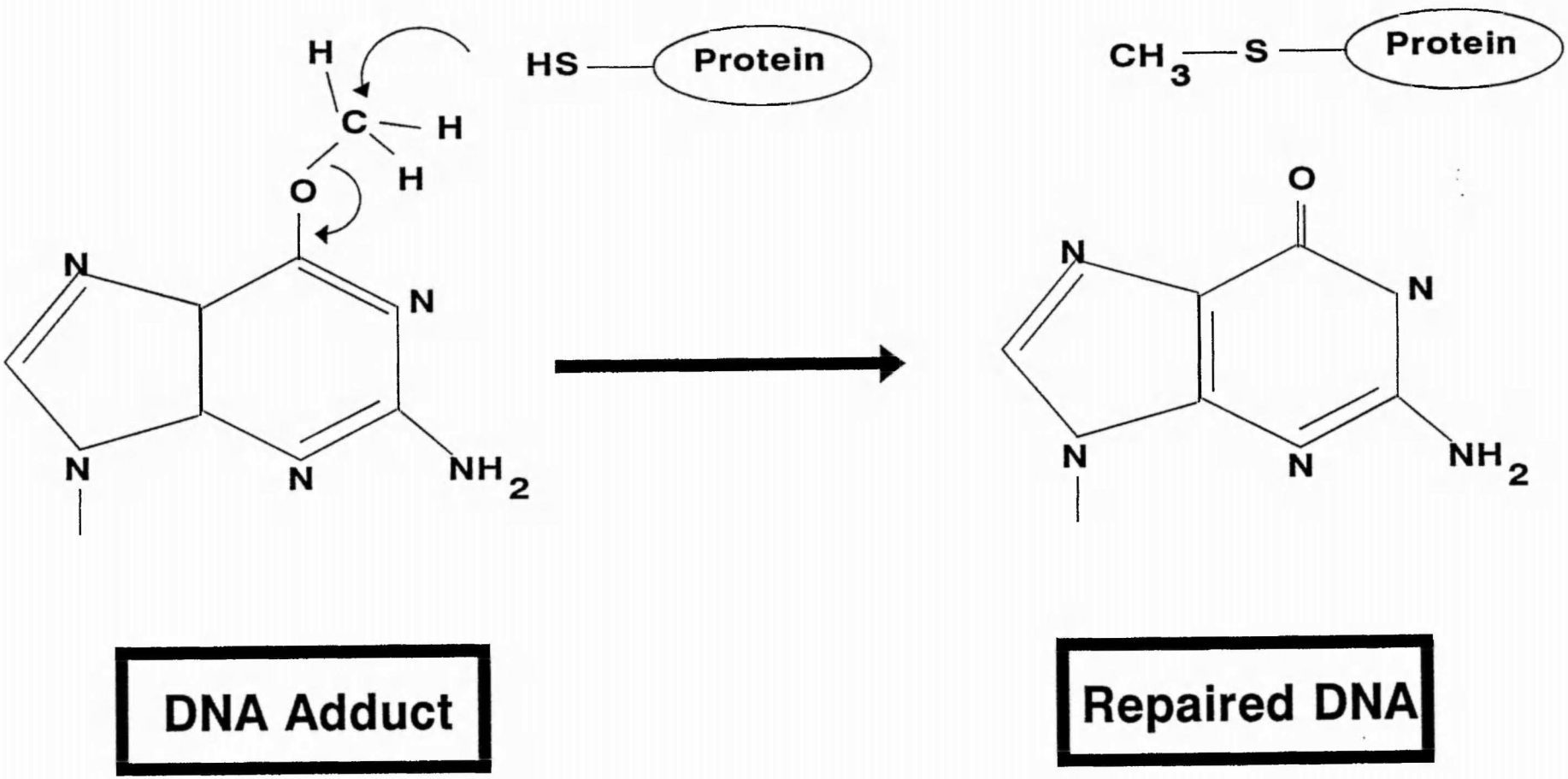


Figure 1.8 Alkyl transfer repair

modified bases that arise by direct alkylation of DNA. Although if misincorporation of an alkylated nucleoside triphosphate occurs then alkyl transfer repair can produce a mutation.

Excision repair operates by removing the modified residue and creating a gap in the damaged DNA (Figure 1.9). The gap can be filled by replication using the unmodified strand as a template. Excision repair is not error free as the repair system requires replication of a template by a polymerase which can make errors. This error frequency is dependent on the different polymerases (Loeb and Kunkle, 1982).

1.2.3.4 Genotoxic carcinogens

Genotoxic carcinogens exhibit both genotoxic and carcinogenic activity. Several polyaromatic hydrocarbons including benzo[a]pyrene as well as alkylation agents such as dimethylnitrosamine are carcinogens that induce a broad range of genotoxic events. These are classified as direct acting. The indirect acting genotoxic carcinogens are metabolically activated to generate electrophilic forms (ultimate carcinogen) either directly or through one or more intermediates (proximate carcinogen) from the parent chemical (precarcinogens). Inorganic compounds can also act as genotoxic carcinogens. Metal ions, (Be^{2+} , Ni^{2+} , Cd^{2+}) act by disrupting DNA replication (Lutz and Maier, 1988). They can reduce template fidelity and promote the incorporation of mis-matching nucleotides.

The expectation that genotoxic activity should predict carcinogenicity is based on the mechanistic similarity between somatic cell mutation and the initiation of carcinogenesis. The conversion of a cellular proto-oncogene to an active oncogene may only require a chromosomal alteration as indicated by the high degree of association between chromosome breakpoints and oncogene locations in tumour cells (Evans, 1986).

Thus three items of scientific information are generally used as criteria to decide whether a carcinogen can be classified as a genotoxic carcinogen:

i) it undergoes bioactivation to reactive electrophilic intermediates eventually producing DNA-adducts in target cells,

ii) it reproducibly gives positive results in a series of *in vitro* genotoxicity tests and

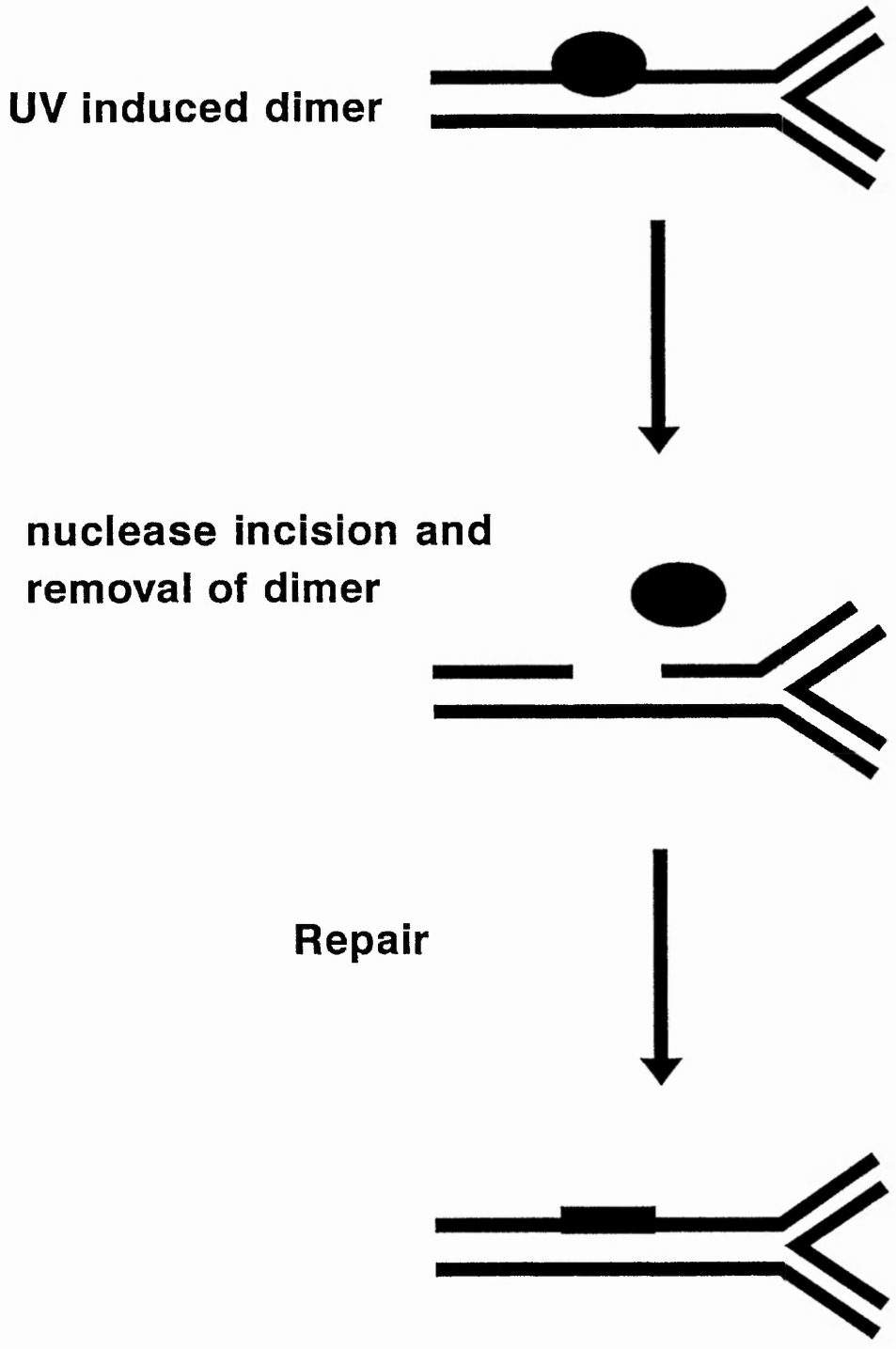


Figure 1.9 Excision repair processes operating in E. coli following damage of DNA by UV radiation

iii) it induces genetic damage of target cells in short term *in vivo* assays.

Co-carcinogens are not carcinogenic but enhance effects of genotoxic carcinogens when administered before, together or at a time when carcinogen damage to DNA is persistent. Formation of tumours by co-carcinogens can occur by any of the following methods:

i) increased uptake or availability of carcinogen,
ii) enhanced metabolic activation of a genotoxic carcinogen or decreased detoxification,
iii) inhibition of DNA repair processes,
iv) increased proliferation of cells with DNA damage, thereby facilitating mutation, codon translocation or amplification (Weisburger, 1983).

1.2.3.5 Non-genotoxic carcinogens

These include chemicals for which the agent is carcinogenic but not mutagenic (Clayson, 1987). There appear to be many carcinogens that induce cancer by a non-genetic mechanism. Diethylhexyphthalate (DEHP) may induce cancer by inducing hepatic hyperplasia and/ or peroxisome proliferation (Butterworth *et al.*, 1984). Methapyrilene is believed to cause liver tumours by inducing increased hepatic cell division and mitochondrial proliferation rather than by a demonstrable genotoxic mechanism (Mirsalis, 1987).

The mechanism of action of non-genotoxic carcinogens is not well understood. In some cases it may involve generation of active oxygen species. Hydrogen peroxide and other oxygen free radicals causing DNA damage either directly or by initiating lipid peroxidation (Rao and Reddy, 1987). A feature common to non-genotoxic carcinogens is that they involve a departure from normal physiological status, a manifestation of which is cellular proliferation (Roe, 1989). Actively proliferating cells may be more at risk of DNA damage or less able to repair such damage than inactive cells. Thus cellular proliferation may act as a factor for predisposition to tumour formation. These carcinogens include cytotoxins, hormones and immunosuppressors. Cytotoxins can act by disturbing the Ca^{2+} homeostasis. An increase in cytosolic Ca^{2+} concentration can activate degradative enzymes such as endonucleases.

The following criteria have been proposed for non-genotoxic carcinogens:

i) it exhibits no significant genotoxic effects in a series of test systems,

ii) it induces specific target lesions characterised by enhanced cell proliferation or sustained cellular hyperfunction/dysfunction in short term animal tests,

iii) it produces hormonal/metabolic/physiopathological effects underlying the occurrence of target lesions in a series of mechanistic studies and

iv) it enhances target tumour occurrence in animals pre-treated with an appropriate initiator.

Table 1.1 is a summary of chemical carcinogens and their mode of action.

TABLE 1.1 Classification of chemical carcinogens

TYPE	MODE OF ACTION	EXAMPLES
Direct acting	Organic electrophile interacting with DNA	Alkylating agents, imines, lactones
Precarcinogens	Require conversion through metabolic activation by host or *in vitro* to electrophile	Aromatics, halogenated hydrocarbons, nitrosamines
Inorganic compounds	Some may be electrophiles but others may lead to changes in DNA by selective alteration in fidelity of DNA replication	Chromium, haematite, lead, nickel
Peroxisome proliferators	Lead to intracellular generation of reactive oxygen species	Chloromethyl phenoxyacetic acid, clofibrate, ethyhexyl phthalate fenobibrate
Cytotoxins	Chronic cell killing leading to increased cell proliferation. Agents capable of causing oxidative stress	Chloroform, peroxides, quinones

Adapted from Weisburger and Williams, 1981.

TABLE 1.1. Classification of chemical carcinogens: (continued)

TYPE	MODE OF ACTION	EXAMPLES
Hormones	Alter endocrine system balance and differentiation. Act as promotors	Androgens, diethylstilboestrol, estradiol
Immunosuppressors	Stimulate virally induced transformed cells	Azathioprine, antilymphocytic serum
Solid state materials	Mechanism unknown, but affects mesenchymal cells. May involve cytotoxicity	Plastics, polymer or metal foils
Promotors	Potentiate effect of genotoxic carcinogens. Enhance development of spontaneously transformed cells	Asbestos, antioxidants, bile acids, saccharin
Co-carcinogens	Not carcinogenic but enhance effects of genotoxic carcinogens when administered before, together or at a time when carcinogen damage to DNA is persistent.	Asbestos, catechol, ethanol, pyrene

Adapted from Weisburger and Williams, 1981.

1.2.4 Mechanism of action of chemical carcinogens

Chemical carcinogens can be classified as either genotoxic (DNA reactive) or non-genotoxic (epigenetic). A genotoxic agent is one capable of altering the information encoded in the DNA either directly or through the formation of a reactive metabolite. These alterations can be point mutations, insertions, deletions or changes in chromosome structure or number (Butterworth, 1989). Genotoxic carcinogens include 2-acetylaminofluorene, dimethylnitrosamine and benzo[a]pyrene. Epigenetic carcinogens do not exhibit genotoxicity as a primary biological activity. However they may yield secondary genotoxic events such as forced cellular growth. These include 2,3,7,8-tetrachlordibenzo-p-dioxin and saccharin (Butterworth, 1989).

The mechanism of carcinogenesis involves interactions between both exogenous (environmental) and endogenous factors. The mechanism proceeds through several stages,

i) initiation,

ii) promotion,

iii) progression.

Initiation is a persistent and heritable alteration of a cell. It creates a potential in the cell or its progeny to undergo malignant transformation if promotion and progression proceed. Initiation is irreversible and is induced by a single dose of carcinogen. Initiation may result from changes in the target cell such as mutation, translocation, amplification (Butterworth, 1989).

Promotion involves the selection and clonal proliferation of initiated cells (Schwarz and Greim, 1986) by compounds that may be endogenous or exogenous in origin. Promotion induces multiplication of initiated cells and thus increases the risk of progression to malignancy. Some carcinogens, including aromatic amines and nitrosamines are capable of both initiation and promotion. These are known as Complete Carcinogens. Incomplete Carcinogens, such as urethane require a promotor for tumour formation. Tumour promotors produce reversible biochemical and cellular responses typical of gene derepression (Schwarz and Greim, 1986). Tumour promotion can be mediated through several mechanisms:

i) differential inhibition of non-initiated cells,

ii) differential stimulation of initiated cells by conferring certain growth advantages on them, or

iii) by disturbing normal intercellular communication by modulation of gap junctions.

Dzarlieva-Petrusevska and Fusening (1985) showed that some tumour promotors could both stimulate cell proliferation and induce chromosomal aberrations. Liver tumour promotors have been shown to produce tumours over long periods of time. Iversen and Astrup (1984) also showed that exposure of the initiator urethane to mice produced skin tumours without the subsequent application of a tumour promotor. For this reason it is no longer possible to refer to chemicals as pure-initiators or pure-promotors.

Progression involves transition of cells from initiated to a malignant phenotype and the further increase in malignancy of tumour cells. It has been demonstrated that readily measurable alterations in the cellular genome are associated with genetic instability of neoplastic cells in the stage of progression (Nicolson, 1987). Karyotypic instability can result in a variety of consequences for the neoplastic cells. These include gene and chromosomal translocations and rearrangements, gene deletions, gene amplification and proto-oncogene activation (Pitot, 1989). Karyotypic instability is unique to the progression stage and is the major factor distinguishing it from the two earlier stages, initiation and promotion.

It thus appears that the formation of a neoplasm follows a common pattern of biological processes which can be triggered and modified by various external and internal stimuli. The changes are subsequently reflected either directly (genotoxic) or indirectly (epigenetic) in alterations to the structure and function of the genome.

Factors affecting the ability of a chemical to cause genetic damage include:

i) metabolic capacity of tissues to which the compound is distributed,

ii) reaction of the compound with DNA,

iii) ability of the cell to repair/amplify the damage and ability of the tissue to express the genetic change.

1.2.5 Detection of genotoxicity by means of short-term tests

Short-term tests (STTs) for genotoxicity detect genotoxic agents and not carcinogens. Evidence suggests, however that genotoxic agents are potential carcinogens (DeMarini *et al.*, 1989). STTs have high statistical power, are almost always replicated, can be performed rather easily under various sets of experimental conditions, are relatively inexpensive and detect a variety of end-points relevant to carcinogenesis.

Criteria important when selecting a test include, reproducibility, rapidity, simplicity, quantification, protocol, cost, manpower, existing database, knowledge of mechanism, specificity and selectivity. The two critical elements of a test are the end-point and the metabolic parameters (Weisburger and Williams, 1981). The end-point should be reliable and of biological significance, while the metabolic parameters should be complementary to others in the group. To increase the probability of correctly predicting carcinogenic effects of compounds with unknown modes of biological activity it is advised that a combination of different tests should be employed.
A summary of STTs follows:

1.2.5.1 Bacterial mutation assays

This is the most widely used assay for determining mutagenicity of chemicals. Because the bacterial strains used in these assays lack the enzymes necessary for metabolising pro-carcinogens to ultimate carcinogens, rat liver extract is added as a crude surrogate of mammalian metabolism. These assays detect point mutations only and measure reverse mutation from amino acid auxotrophy to prototrophy. The bacterial strains used carry base substitution or frameshift mutations in operons coding for synthesis of specific amino acids. The assay determines whether the test chemical can reverse the effect of the pre-existing mutation by introducing a second mutation, either at the structural gene, or at another site on the bacterial chromosome

1.2.5.2 DNA repair tests in cultured mammalian cells

In these tests the occurrence of DNA damage is inferred from the induction of DNA repair by the test agent. The most widely used indicator of repair is the incorporation of radiolabelled nucleotides during gap-filling after excision repair. An important consideration in this assay is which cell line to use. Assays using human fibroblasts and transformed cells require the presence of a microsomal extract as they do not possess the ability to activate proximate carcinogens as does the liver and other organs *in vivo*. Assays using epithelial cells, derived from the liver have overcome this problem as they retain some ability to activate proximate carcinogens. The major advantage of this test is that the phenomena measured is only induced as a result of covalent DNA binding of electrophilic chemical species. Agents which merely intercalate into DNA, such as 9-aminoacridine are inactive in this assay (Bridges, 1976).

1.2.5.3 Budding yeast *Saccharomyces cerevisiae* assay

Yeast cells are unicellular eukaryotes which are easy to grow and whose genetics are well understood. The major advantage of using yeast is the variety of genetic end-points which can be measured. These include point mutation, recombination and both mitotic and meiotic aneuploidy. Yeast assays have a general limitation in that the cell membrane restricts the permeability of certain molecules that might possess genotoxic properties but are unable to reach the target site (Brusick, 1987).

1.2.5.4 Fruit fly Drosophila mutation test

Drosophila melanogaster was one of the first organisms in which mutation induced by chemicals was demonstrated. Knowledge of its genetics is very extensive, and many mutant strains are available for study. The use of *D. melanogaster* in genetic toxicology enables the measurement of induced mutation inherited via the germ cells in an organism with considerable capacity to activate indirectly-acting mutagens and carcinogens. Moreover, a large segment of the genome can be sampled for all major types of mutagenic events.

In Drosophila most of the genotoxic end-points relevant to human hazard can be detected either simultaneously or in separate assays via point mutations, small deletions, duplications, chromosome damage and aneuploidy. Genetic events can also be assayed either in the soma or germ line.

Mammalian-like detoxification pathways have been demonstrated in Drosophila and germ cells have oxidative metabolism at certain stages of development (Edwards and Combes, 1981). Thus Drosophila is convenient for detecting short-lived metabolites because addition of an exogenous metabolising system may be unnecessary. The presence of internal structural differences impose constraints in extrapolation of results from flies to mammals. However, unlike bacteria, Drosophila possesses a cellular and chromosomal organisation more akin to mammals. The life cycle of Drosophila is of convenient duration, short enough to permit rapid analysis of many progeny but long enough to distinguish between chronic, acute and fractionated doses. The major limitation of this test is the lack of quantitative determination of actual dose administered to the flies by feeding (Brusick, 1987). Two important groups of promutagens and procarcinogens are not detectable by the standard recessive lethal test. These are aromatic amines and polycyclic aromatic hydrocarbons.

1.2.5.5 Chromosome damage in cultured mammalian cells

This assay enables the detection of chromosomal mutations by direct microscopical examination of cultured mammalian cells. These tests can be applied to a variety of cell types, including human cells. The most widely used are cell lines in which the capacity for metabolic activation of indirectly acting genotoxins has been lost and S9 is therefore added as for bacterial assays.

1.2.5.6 Gene mutation in cultured mammalian cells

In this assay, point mutation, usually from drug sensitivity to drug resistance, is measured in cultured mammalian cells. Human cells may be used, allowing induction of point mutation to be assessed in a system which is more closely related to human genotoxicology than are the bacterial or fungal systems.

1.2.5.7 Cytogenetic tests in mammals

Rodents are dosed with the test chemical by an appropriate route, and after a given time period, suitable tissues are sampled and chromosome spreads are prepared for examination. The most widely used somatic tissue is bone marrow since this is a rich source of rapidly dividing cells. Germ tissue, usually from the testis, is also used. This assay allows assessment of chromosomal damage in whole mammals after treatment with a test agent (Wyrobek *et al.*, 1983). Since *in vitro* genotoxic agents may or may not exhibit toxicity *in vivo*, it is prudent that such tests be carried out. Assays using whole animals retain the checks and balances afforded by absorption, distribution, metabolism and excretion of foreign compounds, processes which cannot be taken account of in tests conducted *in vitro*, but knowledge of which is essential in attempting to assess the risk.

1.2.5.8 The dominant lethal test in rodents

This assay measures genetic damage to germ cells. It is an *in vivo* test to determine the risk to germ cells from a suspect mutagen (Brusick, 1987). The test is usually conducted after genetic activity has been observed *in vitro* with other mutagenicity tests. A positive result in this assay provides evidence for damage transmitted via the gametes. Dominant lethality may arise from the loss of whole chromosomes or fragments which result from their breakage, for example structural and or numerical chromosomal anomalies or possibly from non-disjunction. It results in the chromosomally deficient embryo dying *in utero*.

This assay is often considered to be less sensitive than other *in vivo* assays. This is often due to inadequate protocols. The failure of some mutagens to give positive results may reflect the true toxicological situation in that germ cells are protected by:

i) a blood/testis barrier,
ii) their metabolic capabilities/incapabilities,
iii) a short half life of the test chemical or metabolites,
iv) some chemicals causing point mutations but not the type of damage detectable in the dominant lethal test.

1.2.5.9 ^{32}P postlabelling analysis

An *in vivo* assay that has gained much importance of late is the ^{32}P postlabelling analysis of carcinogen DNA adducts. This assay has been used to measure formation, removal and persistence of aromatic amine-DNA adducts in rat non-target and target tissues and, to screen chemicals for their capacity to bind to human DNA (Gupta, 1987). This assay has also been applied to the detection of DNA adducts formed by a variety of substances, these include polycyclic aromatic hydrocarbons, azo compounds, mycotoxins, oestrogens and low molecular weight alkylating agents (Gupta *et al.*, 1985). The major limitation of this assay is cost.

1.2.5.10 Conclusion

Although STTs have proven invaluable for detecting genotoxic compounds, mixtures and antimutagens, they require additional development to overcome their many limitations. The animal bioassay is too costly and time consuming to be useful for routine screening except for chemicals with widespread human exposure. STTs are likely to continue to be refined and developed, resulting in STTs that are increasingly more relevant to human mutation and disease (Delehanty *et al.*, 1986). Their utility should not be judged solely against the questionable standard of a rodent carcinogenicity assay.

1.3 BACTERIAL ASSAYS

1.3.1 The *Salmonella*/Microsome assay

The Ames test (*Salmonella typhimurium* mammalian microsomal preincubation mutagenicity assay) is presently one of the best known and most frequently used *in vitro* test systems to detect mutagenic effects of chemicals (Maron and Ames, 1983). The apparent simplicity, sensitivity and accuracy of this method for screening large numbers of chemicals have made it an important tool for the development of safe and useful chemicals. The bacterial tester strains are all derived from *S. typhimurium* LT2 and each strain carries a mutation in one of the several genes which govern the biosynthesis of histidine. Thus these bacteria have a requirement of histidine for growth. The point mutation which ultimately may result from interaction between the reactive chemical and specific DNA sequences restores the function of the histidine operon (Pool and Schmahl, 1987). Cells which sustained mutations that reversed the existing mutation will grow on histidine deficient media. These colonies are known as *his*$^+$ revertants or prototrophs. An increase in revertant colonies with increase in test compound dose indicates a mutagenic response. Mutations are either of the frameshift or base pair substitution type. Most strains contain GC base pairs at the site of histidine mutation and are therefore selective for agents that react predominantly with these bases. A new tester strain, TA102, carries an ochre mutation on a multicopy plasmid and contains AT base pairs at the site of the mutation.

Unlike mammals, these bacteria lack the necessary enzyme systems for metabolising foreign compounds to electrophilic metabolites capable of reacting with DNA. The bacteria are therefore treated with the test compound in the presence of a post-mitochondrial supernatant (S9 or microsomal fraction) prepared from the livers of rats and other experimental animals. The metabolic activity of S9 (predominantly mono-oxygenase activity mediated via the cytochrome P-450 system) is enhanced by treating the rats with a potent inducer of drug metabolising enzymes before they are killed and their livers removed. The S9 is buffered and supplemented with the essential co-factors NADP and glucose-6-phosphate to form S9 mix. By including this metabolising system, many carcinogens otherwise non-reactive are converted into their electrophilic intermediates and thereby detected as mutagens. The capability of the Ames test to detect certain carcinogens can be

enhanced by pre-incubation of the compound and the activation system with the test organism (Yahagi *et al.*, 1975). The addition of cofactors to the S9 mix will further enhance the Ames test detection abilities.

The test is highly sensitive because:

i) a large population of bacteria is exposed to the test agent,

ii) the bacteria divide in the presence of the test compound and the metabolising system,

iii) the bacteria carry a mutation which confers increased cell wall permeability to large hydrophobic molecules,

iv) most of the tester strains are defective in DNA excision repair by virtue of a *uvr* mutation,

v) many of the strains carry a plasmid which enhances error-prone DNA repair,

vi) the histidine mutations contain hot spots highly susceptible to attack by a variety of electrophilic chemicals (Venitt *et al.*, 1983).

1.3.1.1 Bacterial tester strains

The *Salmonella* strains used for mutagenicity strains each contains a different type of mutation in the histidine operon (Maron and Ames, 1983). In addition to the histidine mutation, the tester strains contain other mutations that greatly increase their ability to detect mutagens. The strains and genotypes of bacteria most widely used for mutation assays are listed in Table 1.2.

One mutation, *rfa*, causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria and increases permeability to large molecules, such as benzo[a]pyrene, that do not penetrate the normal cell wall. The other mutation, *uvr*B is a deletion of a gene coding for the DNA excision repair system, resulting in greatly increased sensitivity in detecting many mutagens. The deletion excising the *uvr*B gene extends through the *bio* gene and as a consequence, these bacteria also require biotin for growth. TA102 does not contain the *uvr*B mutation because it was constructed primarily for detecting mutagens that require an intact excision repair system.

TABLE 1.2 Genotypes of the *Salmonella typhimurium* tester strains used for mutagenicity testing

Strain	Histidine mutation	Type of mutation	Main DNA target	Cell wall/ LPS*	DNA repair	Plasmid/ R-factor
TA98	*his*D3052	Frameshift	GC	rfa	*uvr*B	*pKM*101
TA100	*his*G46	Base pair substitution	GC	rfa	*uvr*B	*pKM*101
TA102	*his*G428	Base pair substitution	AT	rfa	Wild-type gene	*pKM*101/ *pAQ*1
TA104	*his*G428	Base pair substitution	AT	rfa	*uvr*B	*pKM*101
TA97	*his*D6610	Frameshift	GC	rfa	*uvr*B	*pKM*101
TA1535	*his*G46	Base pair substitution	GC	rfa	*uvr*B	No
TA1537	*his*C3076	Frameshift	GC	rfa	*uvr*B	No
TA1538	*his*D3052	Frameshift	GC	rfa	*uvr*B	No

* LPS Lipopolysaccharide

Adapted from Maron and Ames (1983); Vennitt *et al.* (1983)

The standard tester strains, TA97, TA98, TA100 and TA102, contain the R-factor plasmid, *pKM*101. The plasmid *pKM*101 carries a gene (*muc*$^+$) which in *rec*A$^+$ / *lex* bacteria participates in SOS DNA repair, a repair pathway induced by DNA damage which confers increased resistance to the lethal effects of many mutagens at the expense of increased mutability (Venitt *et al.*, 1983). Bacteria carrying *pKM*101 have a higher spontaneous mutation rate and mutability than those without the plasmid (*pKM*101$^-$). This plasmid also carries an ampicillin resistant gene. TA102 also contains the multicopy plasmid *pAQ*1, which carries the *his*G428 mutation and a tetracycline resistance gene. These R factor strains are reverted by a number of mutagens that are detected weakly or not at all with the non R-factor parent strains (Levin *et al.*, 1982).

The *his*G46 mutation in TA100 and TA1535 is in the *his*G gene coding for the first enzyme of histidine biosynthesis (Ames, 1976). This mutation substitutes proline for leucine in the wild type micro-organism as below

GGG	for	GAG
CCC		CTC

TA1535 and its R factor derivative TA100 detect mutagens that cause base pair substitutions, primarily at one of these G-C pairs. The *his*D3052 mutation in TA1538 and its R-factor derivative, TA98, detect various frameshift mutagens. Frame-shift mutagens can stabilise the shifted pairing that often occurs in repetitive sequences or hot spots of the DNA, resulting in a frameshift mutation which restores the correct reading frame for the histidine synthesis. The *his*D3052 mutation has the sequence

G C G C G C G C
C G C G C G C G

near the site of a -1 frameshift mutation in the *his*D gene (Levin *et al.* 1982). This mutation is reverted by mutagens such as 2-nitrosofluorene and daunomycin. TA97, a frameshift strain has an added cytosine resulting in a run of 6 cytosines at the site of the *his*D110 mutation (Levin *et al.*, 1982). It is sensitive to all the mutagens that revert TA98, TA1537 and TA1538.

TA104 (*his*G428 *rfa uvr*B/*pKM*101) which is isogenic to the standard tester strains except for the histidine mutation, is sensitive to killing by many mutagens, so that only a narrow range of concentrations are suitable for testing. It does not detect crosslinking agents, ie mitomycin C, which require a *Uvr*B+ background. TA2638 (*his*G428 *rfa*/*pKM*101) detects crosslinking agents but is not as sensitive for detecting oxidative mutagens. Thus a new tester strain was constructed which involved increasing the number of copies of the histidine mutation in the cell by putting the mutated gene on a multicopy plasmid in a $Uvr B^{+}$ background. The plasmid, *pAQ*1, which contained the *his*G gene with the *his*G428 mutation and a tetracycline resistance gene, was constructed and introduced into a cell that carried a deletion of the *his*G gene on the chromosome and also contained the *rfa* (deep rough) marker and the R-factor plasmid, *pKM*101. The strain, TA102 (*his*G428 *pAQ*1/*pKM*101), combined the best attributes of both TA2638 and TA104. TA102 also detected bleomycin which is not detected by either TA104 or TA2638. The *rfa* mutation causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria and increases permeability to large molecules that do not penetrate the normal cell wall. The *uvr*B mutation is a deletion of a gene coding for the DNA excision repair system, resulting in increased sensitivity in detecting many mutagens. TA102 does not contain the *uvr*B mutation because it was primarily constructed for detecting mutagens that require an intact excision repair system. In both *E. coli* and *S. typhimurium*, *pKM*101 increases chemical and spontaneous mutagenesis by enhancing an error prone DNA repair system which is normally present in these micro-organisms. DNA sequence analysis showed that the *his*G428 mutation (contained in TA102 and TA104) contains the sequence

-A-G-A-G-C-A-A-G-**T**-A-A-G-A-G-C-

at positions 839-853, whereas the wild type contains the sequence

-A-G-A-G-C-A-A-G-**C**-A-A-G-A-G-C-

at the same positions. This confirmed the classification of this mutation as an ochre type and the presence of only A-T base pairs in the mutated triplet. Thus mutations that act preferentially at A-T

base pairs will be detected by these strains but will not be detected by the standard tester strains which contain G-C base pairs at the site of reversion.

1.3.1.2 Metabolic activation by S9

Many mutagenic compounds require activation by oxidising enzyme systems before reaction with DNA. For general mutagenicity screening rat liver is the most convenient source of activating enzymes (Maron and Ames, 1983). The liver is quantitatively the most vital organ for the metabolism of foreign compounds. It has high activity of most drug metabolising enzymes, the predominant being cytochromes P-450 monooxygenase. Rat liver can be prepared relatively cheaply and remains active for several months at freezing temperatures. For efficient detection of mutagens requiring metabolic activation it is essential to prepare S9 from induced animals. Aroclor 1254, a mixture of polychlorinated biphenyls is the most commonly used inducer, although other compounds and combinations of compounds are used, including phenobarbital and β-naphthoflavone.

Microsomal preparations can be made from various tissues of other species. S9 preparations from mouse and hamster liver have also been made. Hamster liver extracts are 5-10 times as active as rat liver extracts in acetylating benzidine (Morton *et al.*, 1979). Benzidine is far more mutagenic when tested with hamster S9 than with rat S9 (Prival and Mitchell, 1982). Hamster liver microsomes were about 3 times as efficient as microsomes from 3-methylcholanthrene-induced rats in hydroxylating diacetylbenzidine (Morton *et al.*, 1979). Hamster S9 is more effective in activating mutagens such as N-nitroso compounds, dimethylnitrosamine, diethylnitrosamine, and aromatic amines, 1-naphthylamine, in the *Salmonella* assay (Prival and Mitchell, 1982). The use of uninduced hamster liver S9 instead of Aroclor 1254-induced rat liver S9 enhanced the mutagenic activity of congo red, tryptan blue and direct blue I (Prival and Mitchell, 1982).

The use of 3-methylcholanthrene-induced guinea pig liver S9 enabled detection of the weak mutagenicity of the carcinogen benz(c)acridine. Aflatoxin B1 (AFB1) was more mutagenic after activation by duck liver S9 (Prival and Mitchell, 1982), while vinylidene chloride was shown to be more mutagenic after activation by mouse liver S9 than rat liver S9 (Bartsch *et al.*, 1979). In view

of such interspecies variations in the S9 mix, it would be informative to examine the underlying mechanisms involved in activation or deactivation by S9.

The exclusive use of liver S9 in the Ames test does not allow for tissue-dependent activation of mutagens. A variety of aromatic amines are mutagenic to *S. typhimurium* following metabolic activation by rat intestine S9 (Walters and Combes, 1983). Anderson and colleagues (1985) used rat oesophageal and salivary gland S9 preparations to investigate activation of the oesophageal carcinogen N-nitroso-N-methylaniline. A number of aromatic amines, including benzidine were shown to be more effectively metabolised by bladder S9 (Hix *et al.*, 1983). Bladder S9 has also been used for activation of the carcinogenic aromatic amines, including, 2-naphthylamine and 4-aminobiphenyl to their potent promutagen N-hydroxy metabolite (Poupko *et al.*, 1983).

1.3.1.3 Metabolic activation by bacterial enzymes

The formation of ultimate electrophilic metabolites in the Ames test may include steps that are catalysed by bacterial enzymes. In the absence of these enzymes, intermediates produced by exogenously added mammalian enzyme preparations may not form DNA adducts and as a consequence will not induce mutations (Walters and Combes, 1983).

Nitroreductase deficient strains of TA90, TA100 and TA1538 have been used for studying the metabolism and mutagenicity of nitro carcinogens such as nitroarenes and nitrofurans. These are activated directly to mutagens by bacterial nitroreductases. Bacterial enzymes can also mediate the reduction of high molecular weight azo and nitro compounds with limited cell permeability if a soluble electron carrier such as flavin mononucleotide (FMN) is present (Brown, 1981). In *S. typhimurium* mutagenicity assays which include mammalian S9 preparations for metabolic activation of premutagens, bacterial o-acetyltransferase activity may predominate over similar activity of mammalian enzymes as the low concentration of acetyl CoA in the S9 mix is limiting (McCann and Ames, 1976).

1.3.1.4 Limitations of the assay

Although more than 90% of carcinogens tested are detected as mutagens in the Ames test (McCann and Ames, 1976) the remaining 10% of compounds raise the problem of detecting false negatives. Two classes of false negatives exist:

i) Some carcinogens may elicit their effect via a non-mutagenic mechanism. These include chloroform, saccharin and diethylstilboestrol. They induce tumours in some experimental animals but they are negative in most of the mutagen-based assays. No mutagenicity test system will give a positive result if the carcinogens operate by an epigenetic level.

ii) Most carcinogens and mutagens require metabolic activation to become reactive species. This activation is normally achieved in the liver. When a compound is tested *in vitro*, metabolic activation is stimulated by use of S9 fraction of a rat liver homogenate. The S9 fraction catalyses phase I reactions comprising oxidation of aromatic carbon atoms, of carbon double bonds, O-, S- and N-dealkylations and oxidative deaminations (Testa and Jenner, 1976). It contains little activity of phase II reactions which mostly inactivate reactive intermediates formed during phase I metabolic activation. Such inactivation reactions result in dihydrodiol formation from epoxides by hydratase activity, conjugation reactions such as sulphation, glucuronidation, acetylation and conjugation with glutathione. Due to treatment of the animals with Aroclor 1254 and due to lack of cofactors of the inactivation reactions, activity of these mechanisms of the S9 fraction is relatively low as compared to the phase I reactions. Key activation enzymes may also be absent from the S9 fraction or particular deactivation enzymes may be incapable of generating the active and mutagenic species damaging the DNA and the test will be negative, although the chemical will be carcinogenic or mutagenic in animal experiments (Greim *et al.*, 1980).

The sensitivity of the *Salmonella*/microsome assay makes it a useful tool for rapidly obtaining information about the mutagenic and potential carcinogenic activity of a test compound. The prokaryotic system, however does not detect chromosomal aberrations or other genetic lesions which are detectable only in eukaryotic cells. Greim and colleagues (1980) propose that chemicals be tested in several eukaryotic and prokaryotic *in vitro* tests in combination with testing in animals.

The *Salmonella/* microsome assay with and without metabolic activation is probably the most widely validated and most sensitive system.

1.3.1.5 Relationship between structure and mutagenic activity

There is a strong relationship between chemical structure and mutagenicity in *S. typhimurium* (Shahin, 1987). Shahin showed that the size, position and chemical nature of substituent groups all influence the mutagenicity of monocyclic aromatic amines. He proposed that:

i) there is an inverse correlation between mutagenicity and the size of substituent alkyl or alkoxy groups at the C1 position of 2,4-diaminoalkylbenzenes or 2,4-diaminoalkoxybenzenes,
ii) blockage of one amino group in nitro-p-phenylenediamine by two primary alcohols or blockage of both amino groups each by one primary alcohol, eliminates the mutagenic activity of this compound,
iii) the positions of substituent groups, including electron donating amino and hydroxy groups and electron accepting nitro groups determine the mutagenicity in series of aminonitrophenols and aminonitrobenzene derivatives,
iv) the position of the amino groups in the chemical structure of phenylenediamines strongly influences both mutagenicity and carcinogenicity,

Benzidine in the presence of metabolic activation is capable of reverting *Salmonella* mutagenicity tester strains (Ashby *et al.*, 1980). Ashby *et al.* (1982), studied relationships of mutagenicity, carcinogenicity and chemical structure of various benzidine analogues. They found that neither tetramethylbenzidine nor 9,9'-bijulolidyl were mutagenic in *Salmonella* strains TA98, TA100, TA1535, TA1537 and TA1538. The lack of activity of these compounds was attributed to the inhibition of their metabolic conversion to forms that react with DNA (Ashby *et al.*, 1982). The non-carcinogenicity of benzidine disulphonic acid is believed to be due to its metabolic conversion to non-carcinogenic metabolites. These studies indicate that the mutagenicity and carcinogenicity of these compounds are greatly altered by relatively minor alterations in chemical structure.

Aromatic amines with a methyl group ortho to the amine group are often more carcinogenic than their corresponding unsubstituted amines (Weisburger and Fiala, 1981). Aniline, a monocyclic amine, is not carcinogenic, whereas o-toluidine (2-methyl-aniline) is a rat and mouse carcinogen (Garner *et al.*, 1984). Ortho-methoxaniline, an isomer of anisidine is carcinogenic in both rats and mice, whereas the para isomer is inactive. With cresidines (methylmethoxyanilines) the para isomer which has an ortho-methoxy substitution, was more carcinogenic than the meta isomer with its ortho methyl substitution (Weisburger, 1983). Para-cresidine caused bladder and liver tumours in rats and mice and nasal cavity tumours in rats, the isomer meta-cresidine was solely a rat bladder carcinogen. Further evidence supporting the higher potency of ortho-methoxy substituents over ortho-methyl substituents was obtained by Reid *et al.* (1984) who showed ortho-dianisidine was more mutagenic than ortho-toluidine. Combes and Haveland-Smith (1982) showed o-methyl-p-phenylenediamine to be inactive while its o-methoxy derivative is mutagenic.

In 1948, Miller and Miller, working with butter yellow, showed that the type of alkyl group had a marked effect on the carcinogenicity of the aminoazo dyes and that one methyl group appeared to be essential for activity. The carcinogenicity data on 2'-, 3'- or 4'-methyl, chloro and nitro derivatives of dimethylaminoazobenzene (DAB) showed that activity was determined by the position of the group rather than the type, the activity relationship being 3'>2'>4'.

A number of researchers have shown ways of rendering mutagenic compounds less potent or inactive. The mutagenicity of nitroheterocyclic compounds, in general, reduces on substitution of a ring N-atom with a methyl moiety (Vance *et al.*, 1986). Mutagenicity of a compound can also be reduced or eliminated by blockage of other groups present on the molecules, such as blocking of the amino group of 4,4'-diaminoazobenzene with hydroxyalkyls results in partial or complete loss of activity (Shahin, 1989). Mutagenic activity may also be reduced by sulphonation or substitution of two methyl groups ortho to the amino moiety (Spitz *et al.*, 1950; Ashby *et al.*, 1982). Activity of a potent derivative of DAB, ie MAB, was lost on introduction of a sulphonate moiety at the 4'-position. Direct black 19 and direct black 38 were also rendered inactive upon sulphonation of the p-phenylenediazo moieties (Lin and Solodar, 1988).

Two major obstacles prevent unequivocal conclusions about relationships between chemical structure and mutagenicity. The lack of information on the purity of compounds and on false positive results ascribable to mutagenic contaminants, and secondly, the existence of many contradictions in the scientific literature.

The available evidence supports the following conclusions:

i) structure-activity relationship studies can facilitate development of safe chemical products,
ii) structural similarities do not necessarily predict similar biological activities,
iii) isomers can differ substantially from one another in their mutagenicity and carcinogenicity,
iv) the predictive value of structural features for mutagenicity differs from one chemical class to another.

Despite these limitations, studies of structure activity relationships can contribute to the prediction of mutagenicity of untested compounds, the identification of the basis for false negative and false positive results, the understanding of mechanisms of mutagenesis, the evaluation of the performance of mutational assay systems, the assessment of risks associated with particular compounds and the exploration of the association between mutagenesis and carcinogenesis. There is a strong association between mutagenicity in *S. typhimurium* and chemical structure in various series of monocyclic aromatic amines (Shahin, 1987).

1.3.2 Bacterial Genotoxicity

1.3.2.1 Bacterial differential kill assay

Bacterial differential assays provide one of the most simplest means of detecting chemically induced DNA damage. These tests are based on the principle that the failure to repair DNA damage can result in cell death. Thus strains deficient in DNA repair are killed at concentrations of carcinogen or mutagen at which repair-proficient strains survive.

Historically, the most popular method has relied on diffusion. The test chemical is spotted onto a filter disc placed on the surface of a nutrient agar plate seeded with bacteria (Slater *et al.*, 1971). Such methods are designed for rapidity at the expense of accuracy. Some genotoxins because of molecular size or limited solubility, do not diffuse rapidly. Therefore they fail to show toxicity and give no-test result. Diffusion difficulties can be overcome by using tests carried out in liquid. The most widely used test involves a 'treat and plate' protocol (Green and Muriel, 1976). The test bacteria are exposed to the test agent in liquid (usually buffer or supplemented minimal media). This can be done as a function of time of treatment or as a function of the test concentration. Standard dilution and plating can be used (Rosenkranz and Leifer, 1980) or the Miles and Misra colony count method (Tweats *et al.*, 1981). These tests have the advantage of giving clear quantitative data and are much more sensitive than agar diffusion base methods. A modification of the 'treat and plate' protocol uses growth medium in the place of buffer or unsupplemented minimal medium (Hyman *et al.*, 1980). Thus growing cells are exposed to the test agent. This is a requirement for the demonstration of genotoxicity of a number of chemicals. However, growth of a number of repair proficient and repair deficient strains have to be identical to counter false results due to differential growth.

Alternatively, suspensions of bacteria can be exposed to serial dilutions of test agent and the minimum inhibitory concentration determined, as evidence by the presence or absence of visible growth after a period of incubation (Kada *et al.*, 1980).

A semi-automated method has been published that follows bacterial viability by measuring electrical impedance (Forsythe, 1990). This method overcomes problems associated with McCarroll's group (1981a&b) which measured bacterial growth by turbidity with time after addition of the test chemical to growing cells. Turbidity can be a misleading parameter if used to measure viability, preferential inhibition of DNA synthesis may result in an increase in turbidity due to filamenting cells, even though cell viability is decreased (Leifer *et al.*, 1981).

The differential killing assay can detect a wide variety of different genotoxins. It has a high degree of accuracy in the detection of direct acting mutagens. Despite its simplicity, the differential kill assay has not enjoyed the popularity of the Ames test for routine screening of potential carcinogens and mutagens. Repair deficient bacteria tend to grow poorly in comparison to their repair proficient counterparts. This has been slightly overcome by the use of repair deficient *E. coli* CM871, a *uvr*A *rec*A *lex*A triple mutant that combines extreme repair deficiency with near wild type growth.

The validity of data produced from bacterial repair tests can be compromised by several factors. The most critical and unresolved, is the finding that certain compounds that normally require metabolism to exhibit mutagenicity are direct acting in repair tests. This implies that bacterial repair tests can measure 'non-mutagenic' DNA damage, ie damage that is repairable by error free mechanisms.

Bacterial mutation tests can fail to detect highly toxic mutagens where cell death can mask an underlying mutagenic effect. Such genotoxins are more easily detected by bacterial differential killing tests. In a number of cases, chemicals devoid of any apparent mutagenicity or carcinogenicity are strongly or moderately active in these assays. This may be due to non-covalent DNA binding by some chemicals that may result in lethality for repair-deficient cells, but be of no genotoxic consequence in repair-proficient cells.

1.3.2.2 Bacterial tester strains

The plasmid-containing *E. coli* WP2 series is routinely used in bacterial DNA repair tests. *E. coli* WP2 *uvr*A (*pKM*101) has been demonstrated to detect carcinogens with the accuracy of the *S. typhimurium* TA90 and TA100 combination (Venitt *et al.*, 1983). The use of a strain from the *E. coli* WP2 series is specifically requested by the Japanese guidelines. The tryptophan mutation in the WP2 series is a chromosomal 'ochre' mutation, and the strains should therefore parallel the *Salmonella* tester strain TA104, although little data is available from the screening of chemicals to support this contention. Wilcox *et al.* (1990) showed that a combination of a repair proficient and a repair deficient *E. coli* strain allowed detection of a similar range of oxidative mutagens and cross-linking agents as identified by the strain TA102.

E. coli WP2 has an AT base pair at the critical mutation site within the *trp*E gene. It is excision proficient and thus detects cross-linking agents and carries *pKM*101 plasmid. Some of the features offered by TA102 are also covered by these *E. coli* WP2 strains. From published literature it is apparent that a number of TA102-specific mutagens could be detected in *E. coli* WP2 strains.

Thus different repair deficient strains of the same species respond to a different spectrum of DNA damage. In fact a battery of isogenic strains carrying single repair deficiencies can be used to characterise genotoxins by the type of DNA damage they induce (Green and Muriel, 1976).

Comparisons between the two most commonly used pairs of strains *Pol*A$^+$/ *Pol*A$^-$ and *Rec*A$^+$/ *Rec*A$^-$ show that *Rec*A$^-$ strains appear to detect a broader spectrum of genotoxins than *Pol*A$^-$ strains. Unfortunately many of the repair deficient strains used do not grow as well as their repair proficient counterparts (Kada *et al.*, 1980) This is particularly true of *Rec*A$^-$ strains. The excision deficient strain WP2 *uvr*A (*pKM*101) was the most sensitive *E. coli* strain. A *rec*A strain is totally deficient, lacks recombination and grows poorly with the production of many inviable cells. A *lex*A strain is partially deficient in post-replication repair and almost normal in recombination ability and growth. Both *rec*A and *lex*A mutants are deficient in post-replication repair. A *rec*A *lex*A double mutant was constructed which showed the extreme repair deficiency of a *rec*A strain and showed the almost normal growth of a *lex*A strain (Green and Muriel, 1976). A thymine-requiring

derivative of *uvr*A *lex*A mutant *E. coli* CM611 was obtained by trimethoprim selection. The resulting strain was mated with HfrJC5088 *rec*A and a *rec*A thy$^+$ recombinant selected. The resulting strain *E. coli* CM871 showed virtually the same repair deficiency as the equivalent *uvr*A *rec*A strain WP100, but with the growth characteristics of the *uvr*A *lex*A strain *E. coli* CM611.

Boiteux and Huisman (1989) isolated a formamidopyrimidine-DNA glycosylase (*Fpg*) mutant of *E. coli* K12. The mutation was obtained by cloning a gene conferring resistance to kanamycin into the plasmid copy of *Fpg*$^+$ gene of *E. coli* AB1157. The resulting mutation *E. coli* BH20, *Fpg*$^{-1}$::Knr was then transferred to the bacterial chromosome. The resulting *Fpg*$^-$ mutant had no detectable Fapy-DNA glycosylase activity in crude lysates. The Fapy-DNA glycosylase defective strain was not unusually sensitive to either gamma radiation or to UV-irradiation compared to the wild type strain. Furthermore, the *Fpg*$^-$ mutant did not exhibit increased sensitivity to H_2O_2 or mitomycin C.

TABLE 1.3 Genotypes of the *Escherichia coli* tester strains used for genotoxicity testing

STRAIN	RELEVANT GENOTYPE		REFERENCE
WP2	trp ochre	UV resistant	Bridges *et al.*, 1972
CM871	trp ochre *uvr*A *lex*A *rec*A	Deficient in post-replication repair, lacks recombination	Tweats *et al.*, 1981
AB1157	thr-l leu-6 proA2 *his*-4 argE3 thi-1 lacY1 galK2 ara-14 xyl-33 rpsL31 supE44	8-hydroxyguanine is formed by methylene-blue(MB) light treated DNA and excised by *Fpg* protein.	Czeczot *et al.* 1991
BH20	as AB1157 but *Fpg*-1::Knr	Survival of MB-light treated DNA was nearly the same when transformed in to *uvr*A or *Fpg*-1 single mutants.	Czeczot *et al.* 1991
BH200	as AB1157 but *uvr*A::Tn10	Base excision repair and the nucleotide excision repair pathways mediated by *Fpg* protein and *uvr*A endonuclease are both involved in the elimination of genotoxic lesions formed after treatment with MB-light.	Czeczot *et al.* 1991
BH190	as AB1157 but Fpg^{-1}::Knr *uvr*A::Tn10	Viability is greatly reduced in this strain.	Czeczot *et al.* 1991
BH180	as AB1157 but *nfo- nth-* Fpg^{-1}	Substrate for the *Fpg* protein is DNA damaged by singlet oxygen.	O'Connor *et al.* 1993

TABLE 1.3 Genotypes of the *Escherichia coli* tester strains used for genotoxicity testing (continued)

STRAIN	RELEVANT GENOTYPE		REFERENCE
AB1157-Y11	as AB1157 but mutY zgd::Tn10	8-oxoG, (from oxidative DNA damage) induces targeted G T transversions in *E. coli*.	Moriya & Grollman, 1993
RK1517	as AB1157 but mutS::Tn5	MutY protein (an adenine DNA glycosylase), it removes adenine misincorporated opposite 8-oxoG residues after DNA replication is complete.	Moriya & Grollman, 1993
TT101	as CC104, ara (gpt-lac)5[F'lacI378, lacZ461, proA$^+$B$^+$] but mutM::mini-tet		
RK1517-Y33	as AB1157 but mutY nupG511::Tn10	removes adenine from an A:8oxoG pair *in vitro*:-	Moriya & Grollman, 1993
PQ37	derived from K12	oxyR deletion renders the strain deficient in responding to oxidative stress.	Muller & Janz, 1992
PQ300	oxyR deletion mutant srl$^+$ argE86::Tn10	It is sensitive to H_2O_2, alkylhydroperoxides, superoxide, hydroxyl radical	Muller & Janz, 1992

1.3.2.3 Rapid Automated Bacterial Impedance Technique (RABIT)

The need for improved instrumentation for analysing microbiological samples combining speed and accuracy has resulted in the recognition and the development of impedance techniques. This technique is regularly used in food microbiology.

Impedance is the apparent resistance to the flow of an alternating electrical current. Cultures of micro-organisms bring about changes in the chemical composition of the growth medium through enzymic activity associated with multiplication and metabolism. These chemical changes alter the impedance of the medium and provide an indirect measure of microbial growth and metabolism.

As micro-organisms metabolise they generate new end products in the medium. Generally uncharged or weakly charged substrates are transformed into highly charged end-products, for example proteins are metabolised to amino acids, carbohydrates to lactate and lipids to acetate, which increase the conductivity of the solution. The conversion of one molecule of the non-ionised nutrient glucose to two molecules of the ionised metabolite lactic acid by micro-organisms increases the conductivity of the growth medium. Further metabolic activity, in converting three molecules of oxygen and one lactic acid to three molecules of carbonic acid, further increases the conductance since there are now three ion-pairs where there were originally one. In addition, the smaller bicarbonate ion is more mobile and thus a better electrical conductor than the lactate ion.

The growth of some organisms such as yeasts, does not result in large changes in conductance. This may be due to the fact that these organisms do not produce strongly ionised metabolites, but rather nonionised metabolites, such as ethanol. In addition yeasts have the ability to absorb ions from solution. Hence under certain conditions the conductivity may decrease rather than increase.

Other factors play an indirect role in changes in conductance. Hydrogen ions are nearly seven times more effective as conductors than sodium ions, therefore weakly buffered media would allow a greater conductance change than strongly buffered media.

An advantage of impedance is the ability to multiplex a single measuring circuit, so that a very large number of samples can be measured simultaneously without physical movement of sample, electrodes or measuring system. At Nottingham we used the RABIT (Don Whitley Scientific Ltd., Shipley, UK) an impedance based microbiological growth analyser which is composed of an electrobacteriological interface, controller/analyser and data display. The first component includes the incubation unit which can be set to the proper temperature, and the electrochemical wells with implemented electrodes. This unit transforms the metabolic activity of microbial cells into a measurable quantity. The test micro-organism is in direct contact with the system electrodes. Changes in conductance of the growth medium are directly resultant upon the changes taking place in the bulk electrolyte. Substrates in microbiological growth media, especially those developed for impedance microbiology are generally uncharged or weakly charged but are transformed into highly charged end-products as organisms follow normal metabolic pathways, thus increasing conductivity of the test medium.

The second unit carries out actual measurement of impedance and various analyses, such as determining detection time. The interface to the operator is accomplished by the third unit, in which impedance curves and records are displayed and processed. Microbial metabolism usually results in an increase in both conductance and capacitance causing a decrease in impedance and a consequent increase in admittance which is plotted against time by the RABIT system.

The time required to reach a detectable acceleration in the impedance curve is known as Impedance Detection Time (IDT). This is a function of both initial concentration and growth kinetics of the organisms in the given medium. After the threshold is passed the impedance change with time may be proportional to the number of viable cells. Thus threshold level is a function of the type of organism, type of medium and type of electrodes.

Several factors affect IDT. The time at which acceleration can be seen in the impedance curve depends upon the physical conditions of the system and the microbial characteristics.

i) Microbial Concentration

The higher the cell number the higher the more rapid (earlier) the acceleration.

ii) Generation Times

IDT will correlate with initial concentration only if the generation time of the test population is approximately constant under the experimental conditions.

iii) Electrode Type

The electrical perturbation needed before an impedance change is detected will depend on electrode type. IDT of the same micro-organism in a certain medium will also depend on the type of electrode, their location and configuration.

iv) Concentration of growth medium

At low initial ionic strength, a given change in conductance due to microbial metabolism will be relatively large. At lower concentrations growth media will not have the same levels of nutrients and therefore might result in slower growth.

v) Temperature

As bacterial generation time is effected by temperature, changing temperature will also affect IDT.

1.3.2.4 Calibration curve construction

Prior to adapting an impedance method to estimate numbers of bacteria a calibration curve should be generated. This curve defines the relationship between impedance and the standard method, usually the standard plate count method. In an ideal situation the plot of logCFU/ml versus Impedance Detection Time is a straight line with no scatter along the line (Appendix 1).

1.3.3 The computer automated structure evaluation system

Some chemical groupings, such as an epoxide, are naturally electrophilic and are therefore capable of reaction with DNA. Other groupings such as aromatic nitro groups can be transformed by metabolism to DNA-reactive species. Recognition of these facts led to the electrophilic theory of carcinogenesis (Miller and Miller, 1977). Chemical reactivity can be measured or anticipated by a knowledge of chemistry, and the metabolic transformation of a molecule to an electrophile can be predicted, albeit with less certainty. Recently those electrophilic substructures that have been clearly associated with DNA-reactivity/ mutagenicity/genotoxicity were gathered together in a model chemical (Ashby, 1992; Tennant and Ashby, 1991). The purpose of the model was to enable 'structural alerts' to genotoxicity to be recognised for untested chemicals, with an implication of potential carcinogenicity. As the data accrues the model is enlarged to take account of reactive centres newly associated with genotoxicity.

Rosenkranz and Klopman (1990) have described an artificial intelligence system (CASE) that considers all possible substructures of a chemical and which is then capable of identifying any substructures associated with a particular biological property, such as mutagenicity or carcinogenicity. CASE selects its own description automatically from a learning set composed of active and inactive molecules. First, a learning set of chemicals is entered into the computer and structural fragmentation takes place. Next, a set of biological properties for the learning set of chemicals is entered. Rosenkranz and Klopman currently use a variety of datasets, e.g., for mutagenicity to *Salmonella*, bone-marrow micronucleus activity, carcinogenicity to rats and/or mice, etc. In the case of carcinogenicity, two different, but partially overlapping databases, are used; that of the NTP and that collected by Gold and colleagues. Correlations are then sought between substructures and a particular biological activity such as carcinogenicity. Some substructures may be associated with activity and these are referred to as biophores each having an associated probability (99% being a perfect correlation). Other substructures may correlate negatively with carcinogenicity and these are called biophobes. Correlations are sought individually with each biological activity considered. CASE can be queried regarding the predicted activity of molecules of unknown activity. On the absence or presence of such descriptors, a chemical can be predicted

for activity and projected potency by CASE. A major advantage of the CASE system is that the biophore responsible for a prediction of activity is available for study

Subjecting 1-amino-2-naphthol to CASE analysis revealed the presence of two major biophores which presumably endow the molecule with mutagenic properties. Location of the biophore suggest that sulphonation at any position will result in a decreased likelihood of mutagenicity. The loss of a biophore usually accompanies the formation of a biophobe resulting from sulphonation and this in turn leads to a loss of predicted mutagenicity.

In the future, CASE will be applied to the prediction of carcinogenicity and the identification of fragments that are common to mutagens and genotoxic carcinogens. It is hoped that it will provide insights into the process of mutagenesis and carcinogenesis.

Artificial intelligence systems have the power to increase our understanding of chemical carcinogenesis, but they may come to the wrong conclusions when using a limited database. Aids to the prediction of mutagenicity or carcinogenicity should not act as replacements for the need to understand the mechanisms of the chemical interactions taking place.

1.4 OXIDATIVE STRESS RESPONSE

Oxidative stress is an excess of pro-oxidants in the cell. Active oxygen molecules have been shown to cause damage to DNA, RNA, protein and lipids. It is emerging as one of the most important causative agents of mutagenesis, tumorigenesis and ageing (Farr and Kogoma, 1991). The reactivity of molecular oxygen increases upon acceptance of one, two or three electrons to form, respectively, a superoxide radical, hydrogen peroxide and hydroxyl radicals. The reduction of molecular oxygen is shown as

$$O_2 \rightarrow O_2^{\cdot-} \rightarrow H_2O_2 \rightarrow OH^{\cdot} \rightarrow H_2O$$

The defences against deleterious effects of active oxygen can be divided into preventative and reparative. The former prevents the occurrence of oxidative damage by destroying the offending oxygen species or by limiting the length of certain reactions, such as lipid peroxidation. The latter serves to repair damage caused by offending species that escaped elimination by the prophylactic defence system.

1.4.1 Hydrogen peroxide and DNA damage

Hydrogen peroxide can arise from superoxide anion by catalysed or spontaneous dismutation, and thus may be generated during intracellular autooxidation of many reduced xenobiotics or following enzymic formation of superoxide radical.

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

In addition several oxidases produce H_2O_2 without the intermediate formation of superoxide radical. It is well established that H_2O_2, as well as $O_2^{\cdot-}$ and lipid peroxides, are not sufficiently reactive to attack DNA (Brawn and Fridovich, 1981). However, H_2O_2 is of importance because it can cross cell membranes easily whereas $O_2^{\cdot-}$ cannot. Mello-Filho and Meneghini (1985) carried out experiments in which they exposed cells to a xanthine-xanthine oxidase system. The addition of

catalase provide a protection while superoxide dismutase (SOD) provided no protection against DNA damage.

Of the reactive oxygen species produced by activated neutrophils, H_2O_2 is the most likely to penetrate target cells and to reach the nucleus (Schraufstatter *et al.*, 1988). Shacter and co-workers (1990) have shown that most strand breaks produced in target cells exposed to phorbol ester activated neutrophils result from reactions involving H_2O_2. For these reasons it is important to understand the mechanism behind DNA damage produced by extracellular H_2O_2.

1.4.2 Mechanism of hydrogen peroxide damage to DNA

Hydrogen peroxide has been shown to produce strand breakage in DNA (Aruoma *et al.*, 1991). Ward *et al.* (1987) have demonstrated that numerous double strand breaks are observed at 37°C, whereas at 0°C, only single strand breaks are found. This could result either from differences in the extent of damage inflicted or differences in the activity of repair processes, due to inhibition of cellular reductive processes, or in a combination of these factors. The response curve is non-linear and tends to reach a plateau, indicating the participation of some limiting intermediate in the process (Hoffman *et al.*, 1984). Lipophilic Fe(II) chelators, 1,10-phenanthroline and dipyridyl, inhibit strand break formation in the same way that they inhibit the formation of hydroxyl radical from H_2O_2 and Fe(II) *in vitro*, as detected by the spin-trapping electron spin resonance technique of Mello-Filho and Maneghini (1984). 1,10-Phenanthroline and dipyridyl may enter the cell and chelate the Fe(II) involved in hydroxyl radical generation from H_2O_2. Other biological responses to H_2O_2 resulting in genotoxicity prevented by 1,10-phenanthroline include, chromatid exchange, gene mutation, malignant transformation and cell killing (Mello-Filho *et al.*, 1984b).

A few chelating agents are known, notably desferrioxamine and diethylenetriamine (DETAPAC or DTPA) which are capable of binding ferric iron extremely tightly and with such a shift in redox potential that the iron is biologically not reducible (Gutteridge *et al.*, 1979). Desferrioxamine inhibits superoxide-driven reduction of Fe^{3+} (Halliwell and Gutteridge, 1985) and prevents reduction of Fe^{3+} by ascorbate. However it does not inhibit formation of strand breaks in cells (Mello-Filho and Meneghini, 1985). Although Jonas *et al.* (1989) showed that pre-exposure of a

mammalian cell line (CNCMI-221) to desferrioxamine protected the cells from the toxic effect of H_2O_2.

Mello-Filho and Meneghini (1991) showed that neocuproine, a copper specific lipophilic chelator, which blocks the Cu-mediated Fenton reaction, did not protect cellular DNA from H_2O_2 implying that iron and not copper is the metal involved in mediating DNA damage in most cases. They surmised that iron ions are bound to DNA and that the Fenton reaction generates hydroxyl radical *in situ*.

1.4.3 Superoxide dismutases, protection against DNA damage

There has been much debate in the literature concerning the mechanism of SOD-mediated protection against DNA damage. Different authors have suggested that SOD is protective because a lower steady state concentration of superoxide radical would result in less hydroxyl radical through the iron-catalysed superoxide radical driven Fenton reaction (Freeman and Crapo, 1981; Halliwell and Gutteridge, 1986). Although this is possible, the reaction of $O_2^{\cdot-}$ with Fe^{3+} is relatively slow and reductants such as ascorbate and glutathione that exist at much higher concentrations than $O_2^{\cdot-}$ can substitute for $O_2^{\cdot-}$ in the Haber-Weiss cycle. Thus most of the O_2-mediated toxicity may be due to its direct reactions with critical biomolecular targets. Although the superoxide radical is not very reactive (Sawyer and Valentine, 1981), it can diffuse across a great distance to attack a critical residue before disappearing by means of reactions with non-critical residues. Different direct biological targets for $O_2^{\cdot-}$ have been identified (Fridovich, 1986a).

Iron-sulphur clusters (Fe_4S_4) in proteins are good targets for $O_2^{\cdot-}$. It has been established that in *E. coli* different dehydratases and the tricarboxylic acid enzyme aconitase are preferred targets for $O_2^{\cdot-}$. The superoxide radical causes oxidation of the Fe_4S_4 cluster, resulting in rapid loss of enzymic activity and metabolic disturbances. A transient increase of cell superoxide steady state concentration may inactivate aconitase, which will cause severe impairment of energy metabolism through the inhibition of the Krebs cycle. SOD would protect Fe_4S_4 clusters by scavenging $O_2^{\cdot-}$ at almost diffusion rates.

Another important target of superoxide radical is nitric oxide ($NO^{\cdot}$). Nitric oxide is synthesised by a variety of cell types including, macrophages, endothelial cells and neurons (Marletta, 1990). Superoxide reacts with nitric oxide to form a strong oxidising species, peroxynitrite anion ($ONOO^{-}$). This is protonated to peroxynitrous acid (ONOOH), an unstable species. Beckman and co-workers (1990) have proposed that ONOOH decomposes by homolytic scission to OH' and $^{\cdot}NO_2$.

$$O_2^{\cdot-} + {}^{\cdot}NO \longrightarrow ONOO^{-} \longleftrightarrow ONOOH \longrightarrow OH' + {}^{\cdot}NO_2$$

Peroxynitrite can directly attack protein and non-protein sulphydryls, it induces lipid peroxidation and deoxyribose oxidation by proton catalysed decomposition to OH' and to $^{\cdot}NO_2$, and it causes nitration of tyrosines and phenolic compounds by metal catalysed heterolytic cleavage to nitronium ion (NO^{2+}). Thus peroxynitrite can be a very toxic reactive species and inhibition of its formation could attenuate tissue injury.

SOD exerts part of its antioxidant protection by inhibiting the formation of the cytotoxic $ONOO^{-}$. Hydrogen peroxide, the dismutation product, is less reactive than $ONOO^{-}$ and can be metabolised by specific enzyme systems.

1.4.4 The Fenton reaction

In 1970 Beauchamp and Fridovich presented evidence which suggested that the superoxide radical was acting with hydrogen peroxide (the product of its dismutation) to produce a species with much greater oxidising potential than either of the initial constituents. This new species was presumed to be the hydroxyl radical (OH') generated via a reaction first proposed by Haber and Weiss (1934):

$$O_2^{\cdot-} + H_2O_2 \longrightarrow OH' + OH^{-} + O_2 \qquad \textit{Pathway 1.5}$$

Weinstein and Bielski (1979), reported that the rate constant for this reaction was so slow as to avert biological damage. McCord and Day (1978) showed that the reaction was catalysed by iron:

$$O_2^{\cdot-} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \qquad \textit{Pathway 1.6}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^- \qquad \textit{Pathway 1.7}$$

$$O_2^{\cdot-} + H_2O_2 \rightarrow OH^{\cdot} + OH^- + O_2 \qquad \textit{Pathway 1.5}$$

This combination of reactions is now known as the iron-catalysed Haber-Weiss or as superoxide-driven Fenton chemistry, since Haber and Weiss first postulated all three reactions in 1934.

While nearly everyone agrees that interaction of superoxide, hydrogen peroxide and iron generates a potent oxidant, not everyone agrees on the species generated. In some systems the reactivity of the oxidant differs from that of radiolytically generated hydroxyl radical (Koppenol, 1985; Winterbourn and Sutton, 1986). Koppenol suggested that reaction III may be written as:

$$Fe^{2+} + H_2O_2 \rightarrow FeO^{2+} + H_2O \qquad \textit{Pathway 1.8}$$

The ferryl ion (FeO^{2+}) has oxidant properties similar to, but less potent than the hydroxyl radical ($OH^{\cdot}$). Halliwell and Gutteridge (1990) later postulated that the initial product of the reaction was the oxo-iron complex and that this decomposes to form the hydroxyl radical:

$$Fe^{2+} + H_2O_2 \rightarrow FeO^{2+} \text{ (or } FeO^{3+}) \rightarrow Fe^{3+} + OH^{\cdot} \qquad \textit{Pathway 1.9}$$

Different ligands to Fe^{2+} may stabilise this intermediate, so that little OH' is formed, whereas others destabilise it. Thus iron-EDTA chelates are good sources of OH' in the presence of H_2O_2 whereas haeme rings appear to stabilise ferryl species (Ortiz de Montellano, 1987).

There is no direct evidence that the superoxide radical plays an iron-reducing role in the cell. Indirect evidence obtained with diethyldithiocarbamate, an inhibitor of Cu-Zn SOD, must be taken cautiously since this compound can cause other effects in the cell (Mello-Filho and Meneghini, 1984a). Ascorbate is more abundant than superoxide and has been considered as a more likely candidate for this role. Most ferric chelates react more slowly with H_2O_2 than do Fe^{2+} chelates, so that reducing agents simulate OH' generation. This may occur for ascorbate:

$$Fe^{3+} + \text{ascorbate} \rightarrow Fe^{2+} + \text{semidehydroascorbate} \qquad \textit{Pathway 1.10}$$

Hence, iron (or copper)salt/ascorbate/H_2O_2 mixtures are good sources of OH' radical, and have been used to generated OH' for the determination of reaction rate constants (Halliwell, 1987).

Reaction 1 appears to account for a significant part of the damage that is caused to cells by excess generation of reactive oxygen species (Imlay and Linn, 1988). It is possible that just as oxidative stress causes rises in intracellular free Ca^{2+} ions by interfering with normal Ca^{2+} sequestering mechanisms (Orrenius *et al.*, 1989), oxidative stress also increases the iron ion concentration available in cells to catalyse free radical reactions (Halliwell, 1987). Ferrali and co-workers (1990), showed that iron ion release plays a role in mediating the toxic effects of allyl alcohol in mice.

1.4.4.1 Site Specificity and Fenton Chemistry

High energy irradiation of aqueous solutions produces a substantial yield of OH' which can be removed by addition of OH' scavengers at rates that depend on their concentrations and on the second-order rate constants of these scavengers for reaction with OH'. When these scavengers are added to Fenton chemistry reactions they do not always inhibit OH' radical damage to a biological detector molecule to the extent predicted by their rate constants. This anomaly may be due to site specificity. Since iron ions cannot exist free in aqueous solution, they must either bind to a biological molecule, a buffer or some other constituent of the reaction mix, or else they will precipitate out of solution as polymerised ferric hydroxides and oxyhydroxides. The damage caused by OH' will thus be directed onto the site of iron binding (Borg and Schaich, 1988; Halliwell and Gutteridge, 1990).

Thus a major determinant of the actual toxicity of $O_2^{\cdot -}$ and H_2O_2 to cells may be the availability and location of metal ion catalysts of OH' radical formation. If catalytic iron salts are bound to DNA in one cell type and to membrane lipids in another, excessive formation of H_2O_2 and $O_2^{\cdot -}$ will in DNA cause fragmentation and could initiate lipid peroxidation in the latter. The type of biological damage produced by site specific OH' radical generation will not necessarily resemble that produced by attack of free OH' (e.g. generated by ionising radiation) upon the biomolecule. This has been demonstrated for carbohydrate, nucleic acids (Halliwell and Aruoma, 1991) and proteins (Stadtman and Oliver, 1991).

1.4.5 The role of transition metals in oxidative damage

An important feature of the chemistry of transition metals is their variable oxidation number. Thus, titanium has oxidation numbers of III and IV, copper I and II, and nickel II and III. Possession of oxidation numbers differing by one allows transition metal salts to participate in single electron transfer reactions, so facilitating free radical reactions:

$$Cu(II) + e^- \leftrightarrow Cu(I)$$

$$Fe(II) \leftrightarrow Fe(III) + e^-$$

$$Ti(II) \quad \leftrightarrow \quad Ti(IV) + e^-$$

Thus several transition metals can reduce H_2O_2 in a one electron reaction to generate the hydroxyl radical:

$$M^{n+} + \quad H_2O_2 \quad \rightarrow M^{(n+1)} + OH' + OH^-$$

where M^{n+} can be Ti(III), Cu(I), Fe(II), Co(II) or Ni(II).

In 1962, Dixon and Norman showed that the production of OH' from Ti(III) and H_2O_2 was a fairly simple reaction. By contrast the reactions of Fe(II) and Cu(I) with H_2O_2 is a fairly complex reaction. Only recently has the production of OH' from copper ions and H_2O_2 been confirmed by examining the pattern of damage to purine and pyrimidine bases of DNA (Aruoma *et al.*, 1991). This does not rule out the formation of additional reactive species in systems containing Cu(II) ions. As mentioned previously the reaction of Fe(II) with H_2O_2 produces a ferryl species which can then give rise to OH', a comparable series of reactions might occur in the Cu(II) system, ie an oxo-Cu(II) ion complex might be a precursor of OH' Mixtures of Cu(II) ions and H_2O_2 produce greater DNA base damage than a mixture of Fe(III) ions and H_2O_2 (Aruoma *et al.*, 1991). Thus in terms of its ability to promote DNA damage, Cu(II) is an extremely dangerous metal ion. Cu(II) is also very efficient at promoting peroxidation of certain lipids. These reasons may account for the fact that Cu(II) ions are less extensively used in the human body than Fe(II) ions. They may also explain why proteins able to inhibit formation of reactive species by Cu(II) ions in free solution are so widespread (Halliwell *et al.*, 1987).

The normal intracellular metabolism of iron must play a role in the availability of Fe(II) ions for Fenton reactions in the cell. It has been proposed that iron is normally internalised as Fe(II) ions and that the reductive environment in the cell maintains it in this form (Thorstensen and Romslo, 1990). A cell membrane iron reductase has been found in mammalian cells (Thorstensen and Romslo, 1990) and in yeast which might take part in the process of iron uptake. Iron is stored in ferritin as an Fe(III)-phosphate-oxide complex and apparently the oxidation is processed on the

ferritin surface by a ferrioxidase belonging to the H subunit of the protein (Cheng and Chasteen, 1991). Mobilisation of iron from ferritin requires Fe(III) reduction which may be performed by a cytosolic reductase which can use NADH, NADPH or xanthine as a source of reducing power. Non-enzymic iron mobilisation from ferritin can also be achieved by several reducing agents including superoxide anion and ascorbate. It is therefore assumed that iron circulates inside the cell mostly as Fe(II) ions (Calderaro *et al.*, 1993).

It is clear that Fe(II) ions play an important role in DNA damage produced by reactive oxygen species. This is because DNA has Fe(II) ions as one of its counter ions. If H_2O_2 reaches the nucleus it can react with Fe(II) ions and produce the OH' radical, which in turn attacks *in situ* the sugar or the base, producing strand breaks and base modifications. These DNA lesions may initiate mutation and carcinogenic events. Epidemiological studies have indicated a correlation between body iron stores and risk of cancer (Stevens *et al.*, 1988). It remains to be established why and how Fe(II) ions reach the DNA. Because intracellular iron appears to be tightly regulated it is surprising that it is found so close to such a vital target.

1.4.6 Oxidative damage to DNA

Cells are subjected to oxidative stress when the level of reactive oxygen species exceeds the protective capacity of endogenous antioxidants and radical scavengers. DNA damage (usually measured as strand breakage or chromosomal aberrations) has been almost invariably observed under conditions of oxidative stress (Halliwell and Aruoma, 1991). Table 1.4 shows types of oxidative stress that result in DNA damage.

Oxygen-derived species affect DNA in different ways. Neither superoxide radicals nor hydrogen peroxide cause strand breakage or chemical modification of the purines or pyrimidines in the absence of transition metal ions (Aruoma *et al.*, 1989). Their toxicity *in vivo* is thought to result from their metal ion dependent conversion to hydroxyl radical, which is very reactive towards organic compounds (Steenken, 1989; von Sonntag, 1987). The hydroxyl radical reacts with the DNA bases at diffusion controlled rates by addition reactions: abstraction of hydrogen from the methyl group of thymine also occurs. For example, the hydroxyl radical can add on to guanine at C-4, C-5 and C-8 positions to give OH adduct radicals that can have different fates (Steenken, 1989). Addition of OH' to C-8 of guanine produces a C-8 OH adduct radical that can be reduced to 8-hydroxy-7,8-dihydroguanine, oxidised to 8-hydroxyguanine (8-OHGua), or can undergo opening of the imidazole ring, followed by one-electron reduction and protonation, to give 2,6-diamino-4-hydroxy-5-formamidopyrimidine, usually abbreviated as FapyGua. Similarly, OH' can be added to C-4, C-5 or C-8 of adenine residues: the C-8 OH' adduct radical can be oxidised to 8-hydroxyadenine (8-OHA), or can undergo opening of the imidazole ring, followed by one-electron reduction and protonation, to give 4,6-formamido-4-6-diamino-pyrimidine, (FapyAde).

Pyrimidines are also attacked by OH' to give multiple products (von Sonntag, 1987). Thus, thymine can form cis and trans thymine glycols, 5-hydroxy-6-hydrothymine, 6-hydroxy-5-hydrothymine and 5-(hydroxymethyl)uracil. Cytosine can form several products, including cytosine glycol and 5,6-dihydroxycytosine.

TABLE 1.4 Treatments that induce oxidative stress and DNA damage

OXIDATIVE STRESS
Exposure to ionising radiation
Elevated O_2 concentrations
Exposure to activated phagocytic cells
Exposure to 'redox cycling' drugs
Exposure to cigarette smoke
Exposure to ozone
Direct action of H_2O_2 or organic hydroperoxides
Exposure to auto-oxidising chemicals (adrenalin)
Exposure to xanthine oxidase plus its substrates (xanthine, hypoxanthine)

1.4.7 The role of 8-hydroxyguanine in carcinogenesis

8-Hydroxyguanine (8-OHGua) is one of the major products of base damage when DNA is exposed to physiologically relevant systems producing OH'. It describes the purine base guanine in which the hydrogen atom at C-8 is replaced by an -OH group. There is a direct correlation between the presence of 8-OHGua in DNA and conditions or protocols leading to carcinogenesis (Table 1.5) (Floyd, 1990). These include:

i) ionising irradiation, in the absence of a known chemical carcinogen, that causes cancer induces 8-OHGua formation in cellular DNA;
ii) treatment with $KBrO_3$ causes kidney tumours and causes formation of high levels of 8-OHGua in the target tissue specifically, whereas NaClO or $NaClO_2$, which are equivalent oxidants, do not cause tumours and also do not cause increased formation of 8-OHGua in kidney DNA;
iii) administration of the Fe complex of nitrilotriacetate (NTA) causes kidney tumours and yields a significant increase of 8-OHGua in the target tissue DNA whereas the Na complex of NTA is non-tumorigenic and does not cause increased 8-OHGua in kidney DNA.

It should be noted that despite such strong correlation it is only presumed not proven that conditions that do not cause an oxidant stress yet are carcinogenic do not yield increased levels of 8-OHGua in tissue DNA. It still remains to be proven that 8-OHGua in tissue DNA is a necessary and sufficient condition to cause tumour formation.

TABLE 1.5 8-Hydroxyguanine formation in DNA

Model	Results (8-hydroxyguanine changes)	Reference
Human granulocytes treated with tumour promotor	Several-fold increase in 8-OHGua formation in cellular DNA	Floyd *et al.*, 1986
Ehrlich ascites cells exposed to 4-nitroquinoline-1-oxide	DNA adducts of carcinogen and 8-OHGua increased simultaneously	Kohda *et al.*, 1986
γ-Irradiation of mouse liver	8-OHGua in DNA increased with dose	Kasai *et al.*, 1986
$KBrO_3$ treatment of rats	4-fold increase of 8-OHGua in kidney (target) DNA, NaClO and $NaClO_3$ yielded no change in 8-OHGua	Kasai *et al.*, 1987
2-Nitropropane (2-NP) treatment of rats	2-fold increase of 8-OHGua in liver (target) DNA, 1-nitropropane yielded no change in 8-OHGua	Faila *et al.*, 1989
Chronic ciprofibrate treatment of rats	2-fold increase of 8-OHGua in liver (target) DNA	Kasai *et al.*, 1989

TABLE 1.5 8-Hydroxyguanine formation in DNA

(continued)

Model	Results (8-hydroxyguanine changes)	Reference
Chronic ciprofibrate treatment of rats	2-fold increase of 8-OHGua in liver (target) DNA	Kasai *et al.*, 1989
Choline-deficient diet to rats	large increase of 8-OHGua in liver (target) DNA	Kasai *et al.*, 1989
Carcinogenic Fe-NTA treatment of rats	Fe-NTA caused 2-fold increase of 7-OHG in kidney (target) DNA, non-carcinogenic Na_2-NTA yielded no change in 8-OHGua	Hinrichsen *et al.*, 1990
DNA incubated with asbestos plus H_2O_2	large increases of 8-OHGua in DNA	Kasai and Nishimura, 1984
DNA incubated with Cr(V) plus glutathione	2- to 5-fold increase of 8-OHGua in DNA	Aiyar *et al.*, 1989

The miscoding potential of 8-OHGua was demonstrated by Shibutani and colleagues (1991), using synthetic oligodeoxynucleotides containing a single defined 8-OHdG residue. Chemically modified oligodeoxynucleotides were used to construct primer templates for DNA polymerase studies *in vitro*. DNA synthesis was allowed to proceed in the presence of one of four dNTPs; reaction products were analysed on a denaturing polyacrylamide gel. Kinetic parameters of insertion opposite 8-OHdG and chain extension at the 3' terminus were determined.

Using the Klenow fragment of polymerase I, Shibutani and co-workers (1991) found that dCMP and, to a lesser extent dAMP, was incorporated opposite 8-OHdG. When DNA polymerase a was used, dAMP was primarily incorporated opposite the lesion. Results of studies using DNA polymerase d were similar to those of polymerase a, while results using polymerase b were similar to those obtained with the Klenow fragment (Shibutani *et al.*, 1991). These experiments suggested that the presence of 8-OHdG residues in DNA lead to G:C → T:A transversion mutations in cells. Studies in mammalian and bacterial cells using site specific mutagenesis techniques (Wood *et al.*, 1990; Moriya *et al.*, 1991,; Cheng *et al.*, 1992) confirmed the prediction.

1.4.7.1 Methods of measuring 8-hydroxyguanine and 8-hydroxydeoxyguanosine

8-Hydroxyguanine can be released from DNA by acidic hydrolysis. If enzymic hydrolysis is used, 8-OHGua may be released attached to the 2-deoxyribose sugar. This product is called 8-hydroxy-2-deoxyguanosine (8-OHdG) (Figure 1.10).

Among a wide variety of biomarkers that indicate target organ damage from reactive oxygen species, 8-OHdG has become increasingly popular as a sensitive, stable and integral marker of oxidative stress in cellular DNA. The development of an HPLC technique, coupled with highly sensitive electrochemical detection for the measurement of 8-OHdG has resulted in much information about free radical damage to DNA in intact cells and whole organisms (Floyd *et al.*, 1986). This technique was used to show exposure of numerous cell types to oxidative stress resulted in an increase in the 8-OHdG content of their DNA (Ames, 1989; Floyd, 1990). Oxidative DNA damage has also been measured in human sperm and the amount detected shown to increase in subjects with low intakes of ascorbic acid (Fraga *et al.*, 1990).

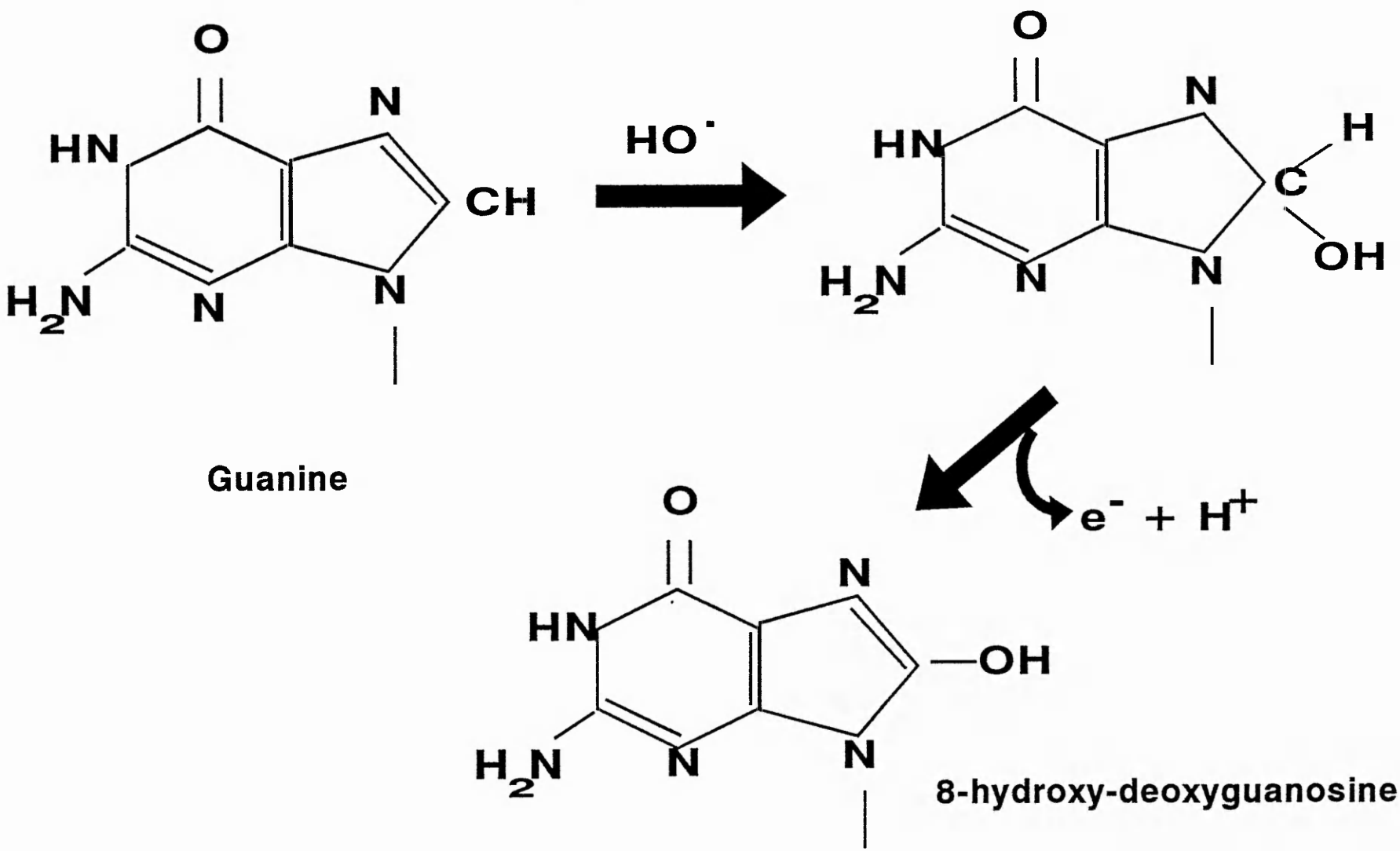


Figure 1.10 Formation of 8-hydroxydeoxyguanosine

These studies have produced qualitative evidence for oxidative damage to DNA *in vivo*. Although this technique cannot be used as a quantitative measure of DNA base damage by reactive oxygen species such as OH'. When OH' attacks DNA bases, radicals are formed that have different fates, depending on experimental conditions. Although attack of OH' on guanine can lead to 8-OHGua, other products such as FapyGua can be formed as well. Thus different amounts of 8-OHGua can result from attack of the same amount of OH' on guanine in DNA. Therefore changes in 8-OHdG levels do not necessarily reflect the amount of free radical attack on DNA. For example, iron ion dependent systems generating OH' cause substantial formation of FapyGua as well as 8-OHGua in DNA (Aruoma *et al.*, 1989), whereas systems containing copper ions and hydrogen peroxide favour 8-OHGua production over FapyGua (Dizdaroglu *et al.*, 1990; Aruoma *et al.*, 1991). Table 1.6 summarises how reaction conditions can alter the end products derived from attack by hydroxyl radical.

Other problems are associated with interpretation of results. An increase in the 8-OHdG content of DNA in a cell might mean an increase in oxidative damage, but may be also due to a decrease in repair. Mitochondria may repair oxidised DNA more slowly than the nucleus showing higher levels of 8-OHGua (Richter *et al.*, 1988). Dead or disrupted cells undergo lipid peroxidation faster than healthy cells (Halliwell and Gutteridge, 1984). It is possible that they also undergo oxidative DNA damage faster so that excretion of DNA base damage products is not necessarily a reflection of the extent of oxidative DNA damage in healthy cells. Routine phenol-based DNA purification procedures can increase 8-OHdG levels in samples exposed to air. While air alone accounts for a significant increase in 8-OHdG levels to DNA samples that have been solubilised in buffers purged with nitrogen (Claycamp, 1992).

TABLE 1.6 Altered end-products derived from attack of hydroxyl radicals upon purine bases in the DNA isolated chromatin

Systems used to generate hydroxyl radical	8-OHA : FapyAde	8-OHGua : FapyGua
H_2O_2 /metal ions /Air saturated solutions		
H_2O_2 /Fe^{3+} /ascorbate	1.1	5.9
H_2O_2 /Fe^{3+} -EDTA	4.2	8.6
H_2O_2 /Fe^{3+} -EDTA /ascorbate	0.5	2.2
H_2O_2 /Fe^{3+} -NTA	1.5	8.3
H_2O_2 /Fe^{3+} -NTA /ascorbate	1.2	5.3
H_2O_2 /Cu^{2+}	18.6	48.4
H_2O_2 /Cu^{2+} /ascorbate	11.1	31.5
Ionising radiation: solutions saturated with		
Argon	0.55	0.57
Air	1.8	3.5
Nitrous oxide	0.8	0.75
Nitrous oxide and oxygen	3.4	4.5

Adapted from Halliwell and Aruoma, 1991.

Although fairly reliable, the HPLC technique may underestimate DNA damage in cells that have been subjected to intense oxidative stress. Extraction of DNA that has undergone extensive oxidative modification and fragmentation may be impaired because of the easy loss of small DNA fragments and of cross-linking of the DNA bases to amino acid residues in nuclear proteins. The efficiency of exonucleases and endonucleases in hydrolysing DNA is greatly affected by modification of the bases (Breimer, 1990). 8-OHGua has been shown to inhibit digestion of dinucleotides by phosphodiesterase. Thus it is not always certain that modified bases are completely hydrolysed from DNA. Finally the HPLC method measures 8-OHdG and not 8-OHGua. Acid pH (used for nuclease P1 digestion) can promote hydrolysis of 8-OHdG to 8-OHGua, causing loss in HPLC detectable material (Frenkel *et al.*, 1991).

Methodologies incorporating the technique of gas chromatography-mass spectrometry (GC-MS) have also been developed for the measurement of free-radical induced damage to DNA (Dizdaroglu, 1985 & 1991). This technique can be applied to DNA itself or directly to chromatin. Base-derived and sugar-derived products and DNA protein cross-links can be chemically characterised and quantified. The technique permits measurement of a large number of products in the same sample of DNA or chromatin in a single analysis. Product yield and the ratio of product yield to one other is dependent on the DNA damaging agents. To analyse the modified bases, DNA is subjected to acidic hydrolysis. This releases modified and intact bases by cleaving the glycosidic bonds between bases and sugar moieties in DNA. A mixture of endo-, exo-nucleases and alkaline phosphatase is used to hydrolyse DNA to nucleosides. After hydrolysis, released bases and nucleosides are derivatised to obtain volatile derivatives. Derivative components are separated on a fused silica capillary column, which is connected to the ion source of the GC-MS. Electron ionisation mass spectra of trimethysilyl derivatives of modified bases and nucleosides of DNA provide considerable structural detail that can be used in identification.

1.4.8 Repair mechanisms of oxidative DNA damage

Two consequences of DNA damage inside cells are of major relevance:

i) the damage can contribute to cytotoxicity (by blocking DNA replication),

ii) the damage can give rise to mutations (by misreading of modified sites of the DNA polymerases or by induction of DNA recombination events).

Both adverse effects are counteracted by the repair mechanisms of the cell. Cells that have been exposed to some agents that produce oxygen radicals exhibit an elevated mutation frequency (Lesko *et al.*, 1980), which may by prevented by radical scavengers (Imlay and Linn, 1988). Conversely, cells deficient in scavengers or reactive oxygen (e.g. superoxide dismutase mutants) or in the ability to repair oxidative DNA damage exhibit elevated spontaneous mutation rates (Ramotar *et al.*, 1991a). McBride and co-workers (1991) reported that single stranded M13mp2 DNA incubated aerobically with Fe^{2+}, a condition that produces free radicals *in vitro*, yielded a broad spectrum of mutations when transfected into *E. coli*. Thus the endogenous production of reactive oxygen species may be a significant source of spontaneous mutations.

Cellular defences against the damaging effects of oxidative stress involve both enzymic and non-enzymic components. The enzymic components may directly scavenge active oxygen species or may act by producing the non-enzymic antioxidants. The protective enzymes are ubiquitous among aerobic organisms. There are four enzymes that provide the bulk of protection,

i) superoxide dismutases, encoded by *sod*A and *sod*B,

ii) catalases, encoded by *Kat*E and *Kat*G

iii) glutathione synthetase, encoded by *gsh*AB

iv) glutathione reductase, encoded by *gor*.

E. coli and *S. typhimurium* do not have NADH-dependent peroxidases specific for hydrogen peroxide, nor do they have glutathione peroxidases.

The reaction catalysed by SOD is thought to occur as follows:

$$Enzyme_{oxidised} + O_2^{\cdot -} \longrightarrow Enzyme_{reduced} + O_2$$

$$Enzyme_{reduced} + O_2^{\cdot -} + H_2 \longrightarrow Enzyme_{oxidised} + H_2O_2$$

There are three types of SOD based upon the metal ligand(s) bound, CuZnSOD, FeSOD and MnSOD. The transition metal facilitates electron transfer. CuZnSOD was first isolated from ox blood by Mann and Keilin in 1938 and is generally not found in bacteria. MnSOD is found both in prokaryotes and eukaryotes, while FeSOD is found primarily in prokaryotes. The reaction between SOD and $O_2^{\cdot -}$ is first order with respect to $O_2^{\cdot -}$. The steady state concentration of $O_2^{\cdot -}$ in a wild type aerobically growing *E. coli* cell is approximately 10^{-9} to 10^{-10} M. In *sod*A*sod*B mutant cells, steady state concentration of $O_2^{\cdot -}$ is approximately 5 x 10^{-6} M. Thus the presence of SOD in the cell reduces the steady state concentration of $O_2^{\cdot -}$ by up to three fold.

A product of $O_2^{\cdot -}$ dismutation is hydrogen peroxide, a reactive species. Thus SOD is not beneficial to the cell unless:

i) the superoxide anion is more toxic than hydrogen peroxide,
ii) hydrogen peroxide disproportionates rapidly to water,
iii) hydrogen peroxide is removed by another method.

Cellular catalases are known to destroy hydrogen peroxide rapidly, the reaction is exothermic and does not require ATP. Catalases therefore provide protection against hydrogen peroxide in an energy depleted cell. *E. coli* cells possess two catalases, HPI and HPII. These are located in the periplasm (HPI) and cytoplasm (HPII), suggesting that sources of hydrogen peroxide may vary during starvation-dependent and starvation-independent oxidative stress (Farr and Kogama, 1991).

Peroxidases can also disproportion hydrogen peroxide but require NADH or NADPH as an electron source. Thus if reducing power is limited, peroxidases are unlikely to provide effective protection.

Glutathione (GSH), an antioxidant, is synthesised by glutathione synthetase. It maintains a strong reducing environment with in the bacterial cell (Loewen, 1984). GSH can react with H_2O_2, $O_2^{\cdot-}$ and $HOO^{\cdot}$ to form a stable glutathione radical ($GS^{\cdot}$). Glutathione reductase transfers an electron from NADPH to the oxidised glutathione (GSSG) to reform reduced GSH (Meister and Anderson, 1983).

$$GSH + HOO^{\cdot} \rightarrow GS^{\cdot} + H_2O_2$$

$$GS^{\cdot} + GS^{\cdot} \rightarrow GSSG$$

$$GSSG + 2NADPH \rightarrow 2GSH + 2NADP^{+}$$

GSH reduces disulphide bridges caused by oxidative stress in proteins. The presence of these bonds, although reversible, can alter protein function. However GSH is not responsible for maintaining the reduced state of most intracellular proteins. Mutants unable to synthesise GSH (*gsh*A$^-$) show no increased sensitivity to H_2O_2 and only slight sensitivity to redox active compounds (Farr and Kogama, 1991; Greenberg *et al.*, 1990). It is possible that other low molecular weight thiols may compensate for the lack of GSH.

An alkyl hydroperoxidase reductase (*Ahp*) found in *S. typhimurium* and *E. coli*, reduces many organic hydroperoxides *in vitro*, including cumene hydroperoxide and t-butylhydroperoxide (Jacobson *et al.*, 1989). Mutants lacking *Ahp* are sensitive to killing by these compounds (Storz *et al.*, 1989). A mutant overproducing *Ahp* was found to suppress the H_2O_2 sensitivity of *E. coli* ZDoxyR cells, suggesting that *Ahp* may act on H_2O_2 directly.

The peroxidases and GSH system require a source of reducing power in order to operate. In *E. coli* the most important catalase, *Kat*G, is inducible, as is MnSOD. Reducing power and the ability to synthesise new gene products may be limited under conditions of oxidative stress. A threshold level may exist where the cell can no longer induce or maintain an effective enzyme defence.

The non-enzymic activities of antioxidant defence systems are due to scavenger activities of small molecules which have high rate constants and thus serve as highly effective scavengers. These include low molecular weight, nonprotein sulphydryl compounds such as glutathione, cysteine and cysteinylglycine. β-carotene, vitamins C and E, chlorophyllin and ergothioneine have all been reported to be effective antimutagens and antioxidants against various chemical mutagens and mutagenic complex environmental and dietary mixtures (Hayatsu *et al.*, 1988).

1.4.8.1 Mutagenicity

Since oxygen radicals cause DNA damage *in vitro* and *in vivo*, and since DNA repair-deficient mutants are hypersensitive to oxidative stress, it is not surprising that oxidative stress leads to mutagenesis (Carlsson *et al.*, 1988; Imlay and Linn, 1987).

It is well established that H_2O_2 induces mutagenicity in *S. typhimurium* and *E. coli* (Carlsson *et al.*, 1988; Imlay and Linn, 1987). At least two mechanisms produce cell damage in the killing of *E. coli* by exogenous H_2O_2. Starved cells can tolerate considerable exposure to H_2O_2 before they are killed. This mode II killing is due to uncharacterised cell damage and exhibits a classical multiple order dose response curve. In mode I killing, actively growing cells are killed by lower, more physiological doses of H_2O_2, particularly if they lack enzymes required for recombination or base excision DNA repair pathways. Similarly as with killing, mutagenicity is biphasic. At mode I, mutagenicity is strongly correlated with SOS induction as measured by induction of l lysogen (Imlay and Linn, 1987). At mode II concentrations, the correlation is weaker. The SOS dependent mutagenesis observed after UV irradiation depends largely upon expression of the *umu*DC operon. Imlay and Linn (1987) have shown H_2O_2 mutagenesis to be *umu*DC-independent in *E. coli*, and that *lex*A(Ind$^-$) mutants are even more sensitive to killing by H_2O_2 than are *rec*A mutants. They concluded that other SOS functions are important for cell survival.

Storz and co-workers (1987), using *oxy*RD mutants in an *S. typhimurium his* reversion assay showed that the pathway for H_2O_2 mutagenicity differed from *E. coli*. When *oxy*RD mutants were grown under aerobic conditions, they exhibited a *His*$^-$ → *His*$^+$ reversion frequency higher than that

exhibited by isogenic wild type cells. Mutagenesis in an *oxy*RD strain carrying *muc*A$^+$ and *muc*B$^+$ on plasmid *pKM*101 is greater than in the wild type strain with the same plasmid. These results suggest that for *S. typhimurium* the products of mucA$^+$ and mucB$^+$ enhance mutagenesis by converting premutagenic lesions to mutagenic lesions. *Muc*A and *muc*B are plasmid encoded analogues of the *E. coli umu*DC genes, which have been shown to be independent of H_2O_2 mutagenesis (Imlay and Linn, 1987). Whether this is due to the difference in mutagenicity between both groups is uncertain.

The nature and pathway of $O_2^{\cdot-}$ mutagenicity are less clear. Since $O_2^{\cdot-}$ is not very reactive towards many biological substrates *in vitro* (Bielski and Shiue, 1978; Bielski, 1985), there remains the important question of how dangerous an increase in $O_2^{\cdot-}$ is *in vivo* and whether endogenously produced $O_2^{\cdot-}$ poses a threat to genetic integrity. $O_2^{\cdot-}$ that is generated by the hypoxanthine-xanthine oxidase system has been shown to cause a significant increase in mutations in *S. typhimurium* TA104, which carries a *uvr*B mutation (De Flora *et al.*, 1989).

The increased mutagenesis observed in cells lacking in SOD activity is oxygen dependent. In anaerobic cultures, the frequency of Thy$^+$ $\rightarrow$ Thy$^-$ mutations is identical for the *sod*A*sod*B double mutant and the wild type strain. When cells lacking in SOD activity are subjected to bubbling of pure oxygen through the culture they display a very high mutation frequency. These results indicate that the increase in spontaneous mutagenesis in cells lacking SOD activity depends on the presence of oxygen and that exposure to increase levels of $O_2^{\cdot-}$ greatly enhances mutagenesis (Farr and Kogoma, 1991).

Unlike H_2O_2 mutagenesis, $O_2^{\cdot-}$ mutagenesis appears to be completely independent of the SOS response. The presence of a *rec*A(*Def*) mutation does not alter the oxygen-dependent mutagenesis enhancement in *sod*A *sod*B mutants (Farr *et al.*, 1986). Farr and co-workers also showed by using *din*D::*lac*Z and *sfi*::*lac*Z fusions (*din*D and *sfi*A genes are repressed by *Lex*A), that SOD-less cells do not display induction of the SOS response.

Evidence shows that superoxide-induced DNA damage cannot be identical to that induced by H_2O_2. Superoxide-induced DNA damage is SOS independent (Farr and Kogoma, 1991). Finally, $O_2^{\cdot-}$ induces synthesis of Endonuclease IV, whereas H_2O_2 does not. It is probable that Endonuclease IV and Exonuclease III do not have identical substrate specificities since overproduction of Endonuclease IV in an *xth*A (encodes Exonuclease III) mutant does not complement the H_2O_2 sensitivity defect of the mutant. Also *nfo* (encodes Endo IV) mutants are sensitive to bleomycin and cumene hydroperoxide, whereas *xth*A mutants are not sensitive to bleomycin (Cunningham *et al.*, 1986).

In 1976, Witkin examined the possible role of the SOS response in oxygen mutagenicity in *E. coli*. The SOS response includes error-prone repair functions that create mutations at the site of DNA lesions as well as the functions that mutate undamaged DNA. It is possible that all DNA contains cryptic lesions, revealed when fixed as mutations in an SOS-induced cell. Active oxygen species are a potential source of such lesions. To determine whether untargeted SOS mutagenesis is oxygen dependent, Farr and colleagues (1991) compared the frequency of $His^- \rightarrow His^+$ reversions in *rec*A441(Tif) strain when cells were grown aerobically and anaerobically. The *rec*A441(Tif) mutation allowed activation of *Rec*A protein at 42°C without DNA damage, leading to an increase in spontaneous mutation rate. The results revealed no reduction in Tif-mediated untargeted mutagenesis in the absence of oxygen. Farr therefore concluded that untargeted SOS mutagenesis was not responding to oxygen dependent lesions.

1.4.8.2 DNA damage and transcription

It has been shown that thymine glycols interfere with transcription by causing RNA polymerase to pause or stop completely at or near the lesion (Byrd *et al.*, 1990). It is probable that single strand breaks, AP sites and many other forms of oxidatively damaged DNA either block RNA polymerase or cause misreading. Both of these effects would result in production of truncated or abnormal proteins. The chaperone proteins, DnaK and GroEL, show increased synthesis after oxidative stress, suggesting the possibility that they serve to handle the increased level of misfolded proteins.

Toxicity induced by severe oxidative damage may result from disruption of transcription as well as from mutagenesis.

1.4.8.3 Membrane damage

Oxidative damage to membranes can arise through either lipid or membrane protein damage. Oxidative stress has been shown to cause peroxidation of lipids both *in vitro* and *in vivo*. Lipid peroxidation entails three steps:

i) initiation,
ii) propagation and
iii) termination.

The peroxidation of lipids generates products which are shorter than the initial fatty acid. The end-products of lipid peroxidation include alkanes, ketones, epoxides and aldehydes. When fatty acids become shortened or charged, their ability to rotate within the membrane is altered. This eventually causes an increase in membrane fluidity, which results in a loss of structural integrity. Structural integrity is required for transport of most nutrients, motility and prevention of osmotic imbalance (Farr and Kogoma, 1991). Because membrane permeabilisation will destroy the proton gradient across the cell membrane, the internal pH will drop, causing the $O_2^{\cdot-} \leftrightarrow HOO^{\cdot}$ equilibrium to shift to the right, which in turn is likely to result in further oxidative damage (Farr *et al.*, 1988).

The peroxidation intermediates and end-products may also be mutagenic. Products such as epoxides and other aldehydes have been shown to be directly reactive with DNA, either by alkylating bases (Segerback, 1983) or by forming intrastrand and interstrand cross-links (Summerfield and Tappel, 1983). Lipid oxidation products also react with and inactivate proteins (Farr and Kogoma, 1991).

The rate of lipid peroxidation is proportional to the number of unsaturated C=C bonds. Bacteria have saturated or monounsaturated fatty acids in their membranes providing an initial defence.

Although it has been shown that *E. coli* fatty acid auxotrophs grown on oleic acid (monounsaturated fatty acid) were more sensitive to killing by oxidative stress than were those grown on saturated fatty acids.

It is also possible that *E. coli* has an inducible membrane repair response. Farr and co-workers (1989) have shown that H_2O_2 disrupts membrane functions at nonlethal doses and that cells which are pre-treated with a low concentration of H_2O_2 acquire the ability to recover rapidly from the loss of membrane function. The peroxide stress response is required for this inducible recovery of membrane function. *Kat*G (under control of *oxy*R) plays a role in this response. It is also possible that the *oxy*R regulated *Ahp* (*ahp*C and *Ahp*F) plays an important role in inducible membrane repair by reducing fatty acid hydroperoxides. The concentrations of H_2O_2 sufficient to induce rapid recovery of transport inhibition has no effect upon transport activities. This would imply that the signal to induce transport recovery does not initiate with damage to the membrane itself.

1.4.8.4 Protein damage

Interactions between oxygen radicals and proteins lead to conversion of proline and arginine residues into carbonyl derivatives. These alterations generally inactivate enzymes and can lead to their targeted degradation. Metal binding sites appear to be more sensitive to attack by active oxygen species. Repair of protein damage appears to be limited to reduction of disulphides and methionine sulphoxides. Reduction of both of these is facilitated by thioredoxin and thioredoxin reductase in an NADPH-dependent pathway (Lunn and Pigiet, 1987). Little is known about the regulation and inducibility of protein repair enzymes.

A protease has been purified from *E. coli* that selectively degrades glutamine. This degradation is ATP-dependent. The role of this degradation uncertain, it may be to expedite recycling amino acids, prevent accumulation of proteins that cause induction of the heat shock response or accumulation of glutamate owing to inactivation of a high proportion of the total glutamine synthetase. Regulation of these proteases is also poorly understood, although Constitutive (*Oxy*R) mutants show a two fold increase in protease activity, suggesting a possible role for *oxy*R in induction (Lin and Sancar, 1989).

1.4.9 Repair pathways

1.4.9.1 DNA glycosylases

Damaged DNA bases are removed by DNA glycosylases, of which there are two functional types. Simple DNA glycosylases hydrolyse the N-glycosylic bonds between a damaged or inappropriate base and the deoxyribose sugar to releases the base and the produce an unmodified AP site. A second type of glycosylase not only hydrolyses the N-glycosylic bonds of its target damage, but also contains β-lyase activity that cleaves the resulting AP sites by β-elimination. This lyase reaction produces a 3'-terminal α,β-unsaturated aldehyde, which requires further processing by enzymes that remove the blocking group. The intact AP sites produced either by the action of simple glycosylases, or through the slow hydrolytic loss of both undamaged purines and the faster elimination of many damaged bases are incised by hydrolytic (Class II) endonucleases, which cleave the phosphodiester bond immediately 5' to the AP site. These enzymes produce normal 3'-hydroxyl termini that can be used for DNA repair synthesis. Damaged bases as well as AP sites may also be removed by other DNA repair pathways, such as the *Uvr*ABC complex in *E. coli* or recombinational DNA repair systems. The relative efficiencies of these latter systems compared with base excision pathways is still unclear.

1.4.9.2 *E. coli* endonuclease III

Endonuclease III was discovered in cell free extracts of *E. coli* as an activity that cleaves DNA irradiated with X-rays or high doses of UV light (Radman, 1976). The UV lesions recognised by endonuclease III were not cyclobutane dimers, and it was also noticed that the enzyme cleaves DNA treated with osmium tetroxide. Osmium tetroxide produces thymine glycol, which is also formed by X-rays (von Sonntag, 1987; Wallace, 1988) and in low yield by UV light (Hariharan and Cerutti, 1977). Endonuclease III was subsequently shown to act as a DNA glycosylase that releases free thymine glycols from osmium tetroxide treated DNA (Demple and Linn, 1980). Together with the AP cleaving activity of endonuclease III this result suggests a two step DNA cleavage mechanism:

i) removal of thymine glycol by the DNA glycosylase function,

ii) followed by cleavage of the resulting abasic site by the AP endonuclease function.

The glycosylase activity of endonuclease III also removes other oxidative products of thymine and dihydrothymine, and urea or N-substituted urea derivatives (Wallace, 1988).

The endonuclease III structural gene *nth* was cloned by a mass screening procedure and *E. coli* mutants lacking endonuclease III activity were constructed by gene targeting (Cunningham and Weiss, 1985). *Nth*$^-$ mutants were not hypersensitive to oxidants such as hydrogen peroxide or X-rays, or to any other DNA damaging agents (Cunningham and Weiss, 1985). They also showed that genetic modification of endonuclease III did not alter the general mutation rate in *E. coli*. Other *E. coli* DNA repair enzymes that can recognise and repair lesions such as thymine glycols (e.g. *Uvr*ABC) may replace endonuclease activity in *nth*$^-$ cells.

Cunningham and colleagues (1986), purified endonuclease III and have shown it to contain an iron-sulphur (4Fe-4S) cluster. The iron-sulphur cluster did not appear to be redox active and its relation to the endonuclease III mechanism was unknown. T4 endonuclease V, a β-lyase, did not contain iron or other tightly bound metals (Morikawa *et al.*, 1992), thus it is unlikely that the iron-sulphur cluster plays a critical role in the β-elimination reaction.

Activities similar to endonuclease III have been identified. Gossett and co-workers (1988) identified an enzyme from *Saccharomyces cerevisiae* which cleaves oxidised DNA substrates containing thymine glycol to produce 5'-phosphoryl termini and modified unsaturated 3'-deoxyribose residues.

1.4.9.3 FAPy glycosylase/ mutM protein

This enzyme was initially detected in *E. coli* extracts as a DNA glycosylase that released ring-opened guanines resulting from alkali treatment of N-7-[3H]methylated DNA: methyl-FAPyGua residues (Chetsanga and Lindahl, 1979). The *Fpg* gene coding for the enzyme was cloned from *E. coli* by Boiteux and colleagues (1987). By 1990, Boiteux's group had constructed enzyme deficient strains. The catalytic site(s) of the *Fpg* protein has not been established. Using atomic

spectroscopy, the enzyme was shown to contain one zinc atom per molecule (Boiteux *et al.*, 1990). Amino acid analysis revealed a single putative zinc finger motif of the CC/CC type located near the carboxyl terminus (Grollman, 1992) where a Zn^{2+} ion may be tetrahedrally co-ordinated between the sulphur atoms on the four cysteine residues. Site directed modification of the *Fpg* gene shows that the putative zinc finger of *Fpg* protein is required for DNA binding (Tchou *et al.*, 1991).

Like endonuclease III, the *Fpg* protein incises AP sites by a β-elimination mechanism (O'Connor and Laval, 1989). *Fpg* also removes the unsaturated 3'-deoxyribose, yielding 3'-phosphate termini (Bailly *et al.*, 1989). The *Fpg* protein also has dRPase activity. This results in removal of 5'-terminal deoxyribose-phosphates produced by incision of AP sites by class II AP endonucleases. The *Fpg* protein is the major EDTA-resistant dRPase activity in crude extracts.

Fpg has a relatively broad substrate specificity. In addition to the ruptured purine ring FapyGua (Boiteux *et al.*, 1992) and FapyAde (Breimer and Lindahl, 1984), the enzyme removes the oxidised ring-closed purines 8-oxo-Gua (Tchou *et al.*, 1991) and to a lesser extent 8-oxo-Ade (Boiteux *et al.*, 1992) from DNA. This additional specificity accounts for the 8-oxo-Gua endonuclease activities in *E. coli* identified by Chung (1991a&b).

Mutants lacking *Fpg* protein are not hypersensitive to hydrogen peroxide or γ-rays (Boiteux and Huisman, 1989). *Fpg*$^-$ strains were also found to be proficient in the repair of pBR322 plasmid DNA damaged *in vitro* by methylene blue plus visible light (Czeczot *et al.*, 1991), a treatment that generates large amounts of 8-oxo-Gua in DNA (Epe *et al.*, 1988). However, when a *Fpg* mutation was combined with a mutation in the excision repair pathway (*Uvr*A$^-$), the double mutant was deficient in repairing plasmid DNA treated *in vitro* with methylene blue plus visible light (Czechot *et al.*, 1991). The *uvr*A$^-$ single mutants, like the *Fpg* single mutants, were proficient in repairing the damaged pBR322 DNA. Thus, at least two repair pathways act on purine oxidation products such as 8-oxo-Gua and the Fapy lesions.

Recent studies show that the previously identified *mut*M gene of *E. coli* is identical to the *Fpg* gene (Michaels *et al.*, 1992). *E. coli* with a *mut*M phenotype are characterised by an increased frequency

of G:C → T:A transversions (Cabera *et al.*, 1988), expression of the Fpg protein in a *mut*M background suppresses this mutagenesis (Michaels *et al.*, 1992). Inactivation of the *Fpg* gene, creating the *mut*M phenotype, leads to G:C → T:A transversions. The endogenous mutagenesis may be due to the formation of 8-oxo-Gua during normal cell growth, because site specifically located 8-oxo-Gua residues target misincorporation of dAMP by DNA polymerases *in vitro* (Shibutani *et al.*, 1991) and the production of G:C → T:A transversions *in vivo* (Wood *et al.*, 1990). This is consistent with studies of the mutagenic specificity of 8-oxo-dGTP during DNA synthesis *in vitro* (Cheng *et al.*, 1992). However, methylene blue plus light must produce other mutagenic guanine lesions because single stranded DNA damage by this agent yields predominantly G → C transversions (McBride *et al.*, 1992).

Although this suggests a role for *Uvr* excision repair in repairing 8-oxo-Gua (Czechot *et al.*, 1991), this may not extend to the lesions generated by endogenous DNA-damaging agents, in light of the strong mutator effect of *mut*M mutations. The possible contribution of Fapy lesions to oxidative mutagenesis caused by endogenous or exogenous agents is also unsure. Finally, if the mutagenic lesions (8-oxo-Gua) that elevate the mutation rate of *mut*M$^-$ strains are due to oxidation damage, a normal mutation rate would be expected for the strains grown anaerobically. Such experiments have yet to be reported.

1.4.9.4 Hypoxanthine glycosylase

The deamination of adenine in DNA forms hypoxanthine which can pair with cytosine to cause A:T → G:C transition mutations. An activity that excises hypoxanthine from DNA-containing hypoxanthine residues was found in extracts of *E. coli* and mammalian cells (Myrnes *et al.*, 1982). Hypoxanthine DNA glycosylase has been partially purified from *E. coli* and from calf thymus. Both activities remove the deaminated adenines from single I:T base pairs *in vitro*. In *E. coli*, the enzyme also repairs transfected M13 RF DNA that contains I:T base pairs (Hill-Perkins *et al.*, 1986). The partially purified calf thymus protein removes hypoxanthine more efficiently from I:T base pairs than from I:C base pairs present in synthetic oligonucleotides. This enzyme does not act on other mismatches, such as G:T, A:G or A:C. Whether this glycosylase recognises lesions other than hypoxanthine remains unknown.

1.4.9.5 Apurinic/apyrimidic endonucleases

1.4.9.5.1 *E. coli* exonuclease III

E. coli exonuclease III has several enzymic activities. Richardson and Kornberg, 1964, first identified the 3' → 5' exonuclease activity that degrades double-stranded DNA. It has 3'-phosphomonoesterase and 3'-phosphodiesterase activity that removes 3'-phosphoglycoaldehyde esters and 3'-phosphoglycolate esters (Demple and Linn, 1980). Exonuclease III is the main AP endonuclease activity in *E. coli* extracts and can cleave the phosphodiester bond just 5' to the AP site, dissimilar to the β-lyase activities of endonuclease III and Fpg. The AP endonuclease referred to as endonuclease VI is identical to exonuclease III. Exonuclease III also has RNAse H activity of undetermined significance (Demple and Linn, 1980).

Exonuclease III removes blocks 3'-termini produced *in vivo* by hydrogen peroxide radicals (Demple *et al.*, 1986). *xth*A$^-$ mutants, which have no exonuclease III activity, are extremely sensitive to hydrogen peroxide (Demple *et al.*, 1986), but not to γ-rays (Cunningham *et al.*, 1986). The hypersensitivity to hydrogen peroxide is due to unrepaired DNA strand breaks in chromosomal DNA that accumulate at a 20-fold greater rate in *xth*A$^-$ mutants compared to wild type cells (Demple *et al.*, 1986). These breaks contain 3'-ends that do not support DNA repair synthesis by DNA polymerase I *in vitro*, but which are activated *in vitro* by exonuclease III (Demple *et al.*, 1986). Repair was found not to be due to the 3'-phosphomonesterase activity of exonuclease III since another 3'-phosphatase (T4 polynucleotide kinase) had little effect (Demple *et al.*, 1986). It was therefore concluded that the 3'-blocks caused by hydrogen peroxide are diesters which are removed *in vivo* by exonuclease III. Exonuclease III can also remove the α,β-unsaturated 3'-deoxyribose products generated by endonuclease III and T4 UV endonuclease (Mosbaugh and Linn, 1982). Exonuclease III activates DNA synthesis on DNA damaged *in vitro* by bleomycin which contains 3'-phosphoglycolate esters and 2-deoxyribose-pentose-4-glucose.

Exonuclease III is expected to be involved in the repair of most lesions that are recognised by DNA glycosylases. It accounts for approximately 85% of the endonuclease activity present in *E. coli* extracts. Cells deficient in exonuclease III activity are mildly hypersensitive to mono-functional

alkylating agents (Cunningham *et al.*, 1986) which produce targets for spontaneous depurination and DNA glycosylases (Sakumi and Sekiguchi, 1989).

1.4.9.5.2 *E. coli* endonuclease IV

Endonuclease IV, an *E. coli* enzyme, removes 3'-blocking damages and attacks AP sites in DNA. It was identified when crude extracts of *xth*$^-$ mutants were found to contain residual AP endonuclease activity that had different properties from exonuclease III (Ljungquist, 1977). It was shown to cleave DNA containing AP sites but not UV-induced damages. Mass screening yielded the gene *nfo* coding for endonuclease IV, and *nfo*$^-$ mutants were created by gene disruption (Cunningham *et al.*, 1986).

Mutants that lack only endonuclease IV were found to be specifically hypersensitive to the oxidants t-butylhydroperoxide and bleomycin. Deficiency in endonuclease IV further sensitised exonuclease III-deficient *E. coli* to hydrogen peroxide and mitomycin C, and only the double mutants exhibited increased sensitivity to γ-rays (Cunningham *et al.*, 1986). *In vitro* evidence suggests that endonuclease IV repairs blocked 3'-termini and constitutes further defence for AP sites.

Endonuclease IV was subsequently found to be inducible by agents that generate superoxide radicals (Chan and Weiss, 1987). Like exonuclease III, it possesses AP endonuclease, 3'-repair diesterase and 3'-phosphatase activity (Levin *et al.*, 1988).

This enzyme contains essential metal ions bound tightly to the protein. Atomic absorption spectroscopy revealed that endonuclease IV contains, on average, 2.4 atoms of zinc and 0.7 atoms of manganese (Levin *et al.*, 1991). At this stage it does appear the protein contains discrete metal-binding sites. Activity was lost after removal of all metals, except for one tightly bound zinc. Enzyme activity was restored by the addition of manganese or cobalt but not by zinc alone. This suggests that endonuclease IV has a metal binding site that is not satisfied by zinc, and which could be regulatory in nature (Levin *et al.*, 1991).

Both endonuclease IV and exonuclease III can activate DNA repair synthesis *in vitro* to the same extent in hydrogen peroxide-damaged chromosomal DNA (Demple *et al.*, 1986). The

overproduction of endonuclease IV in *xth*A$^-$ cells does not restore hydrogen peroxide resistance (Ramotar *et al.*, 1991b). It appears that exonuclease III and endonuclease IV recognise specific DNA damages *in vivo*. Levin demonstrated this in 1991 by showing endonuclease IV *in vitro* activated primer termini in chromosomal DNA from bleomycin-treated *nfo*$^-$ cells more efficiently than exonuclease III.

1.4.9.6 Nucleotide excision repair

Nucleotide excision repair contributes to the repair of some oxidative DNA damages. In *E. coli* this pathway is initiated by the *Uvr*ABC complex, which binds at the DNA lesion and cleaves the damaged strand nine nucleotides 5' to the damaged base and two to four nucleotides 3' to the damage, depending on the type of lesion (Sancar and Sancar, 1988; Grossman and Yeung, 1990). Displacement of the damaged oligonucleotide and DNA repair synthesis mediated by *Uvr*D and DNA polymerase I, following by ligation to seal in the newly synthesised DNA, completes the repair process. The *Uvr*ABC complex recognises a wide range of DNA lesions such as

i) bulky damages, cyclobutane and 6,4-pyrimidine-pyrimidone UV photoproducts, N-2-acetylaminofluroene adducts,
ii) cross links, mitomycin C,
iii) AP sites.

Earlier studies pointed to a role for nucleotide excision repair in removing oxidative damages from DNA. γ-Irradiated ϕX174 RF DNA was less efficiently replicated in excision repair-deficient mutants (*uvr*A$^-$ or *uvr*C$^-$) than in wild type *E. coli* (Nabben *et al.*, 1984). The apparent non-viability of *uvr*A$^-$ *xth*$^-$ *nfo*$^-$ triple mutants suggests that the *Uvr*ABC complex can repair some of the oxidative damages recognised by exonuclease III. It is unknown whether the triple mutants which survived under anaerobic conditions were viable aerobically. The *uvr*A$^-$ *xth*$^-$ *nfo*$^-$ triple mutants were viable aerobically in a uracil glycosylase-deficient background, or if the *sul*A gene was mutated (Foster, 1990). Foster showed that the AP sites formed by uracil excision were lethal because of constitutive activation of the *sul*A cell division inhibitor.

In vitro studies demonstrated that *Uvr*ABC can recognise both AP sites and thymine glycols in a 49 base pair DNA fragment (Lin and Sancar, 1989) or on PM2 DNA (Kow *et al.*, 1990). Two other oxidative lesions, 8-OHGua and FapyGua, which are produced by the singlet oxygen generator methylene blue during exposure to visible light, also seem to be substrates for the *Uvr*ABC complex *in vivo* (Czechot *et al.*, 1991). These lesions are substrates for the Fpg protein also (Boiteux *et al.*, 1992). Single mutants lacking either repair pathway have normal resistance to methylene blue plus light and to many other redox agents (Boiteux and Huisman, 1989; Czechot *et al.*, 1991). Thus the *Uvr*ABC complex participates in the repair of certain oxidative lesions, but this nucleotide excision repair may be ancillary to the base excision pathways initiated by endonuclease III of Fpg protein.

1.4.9.7 Post-replication repair

Oxidative lesions that block replication can cause DNA synthesis to resume 1000 to 42000 nucleotides downstream, leaving long single-stranded interruptions in the daughter DNA strand, referred to as daughter-strand gaps (Rupp and Howard-Flanders, 1968). These gaps can be repaired in a *Rec*A-dependent post replication repair pathway in *E. coli*. This may also occur in the repair of x-ray induced double strand breaks (Smith and Meun, 1970). The restoration of DNA after double strand breakage requires the presence of a duplicate genome, which indicates that the process involves genetic recombination.

The *E. coli* RecA protein has at least two functions in post replication repair and genetic recombination:

i) an ssDNA dependent ATPase which allows RecA to polymerise on ssDNA prior to formation of complexes with dsDNA,

ii) a recombinase that allows RecA to transfer a DNA strand from one homologous partner to another to produce heteroduplex DNA (Dasgupta *et al.*, 1981). The cross over point is subsequently resolved to produce the independent heteroduplexes (Tsaneva *et al.*, 1992). This pathway is also dependent on the *rec*BCD and *ruv*ABC gene products.

A second pathway (*Rec*F) in *E. coli* can repair daughter strand gaps in duplex DNA (Sharma and Smith, 1985). This pathway requires the functions of at least ten genes including *rec*A, F, J, N, O, Q, *ruv*ABC and *uvr*D (West, 1992). Six of these genes *rec*A, N, Q, *ruv*A and *uvr*D are inducible by DNA damaging agents (Tsaneva *et al.*, 1992) and are in the SOS pathway controlled by *Lex*A protein and *Rec*A (Thliveris *et al.*, 1991).

Yeast may have mechanisms operating in post replication repair similar to those of *E. coli*. Basic biochemical considerations indicate the fundamental utility of recombinant repair, and physical evidence for such repair in mammalian cells may exist in the form of damage-induced sister chromatid exchanges (West, 1992). However, the biochemistry of this process in mammalian cells remains largely unexplored.

1.5 DIETARY ANTIOXIDANTS

Many defence mechanisms within the cell have evolved to limit levels of reactive oxidants and the damage they inflict. These affects vary and include the direct inhibition of oxidant-induced damage, inhibition of oxidant-induced mitogenesis, protection against oxidant-induced degradation of immune surveillance mechanisms and possibly inhibition of metastases. Among the defences are enzymes such as SOD, catalase and GSH peroxidase as well as the dietary antioxidants, ascorbic acid, α-tocopherols, β-carotene, glutathione and ubiquinol (Bagchi *et al.*, 1993).

The term antioxidant may be broadly defined as a substrate which when present at low concentrations compared with those of an oxidisable substrate (fats, lipid components, protein, carbohydrates and DNA) significantly delays or prevents oxidation of the substrate (Halliwell and Gutteridge, 1990). Antioxidants act at different levels in the oxidative sequence involving lipids.

Antioxidants may act by:

i) decreasing localised oxygen concentrations,
ii) preventing first chain initiation by scavenging initiating radicals such as hydroxyl radicals,
iii) binding metal ions in the forms that will not generate the lipid peroxidation initiating species, such as hydroxyl radical, ferryl radical or Fe^{2+}-Fe^{3+}-O_2 complexes and/or will not decompose lipid peroxides to peroxyl or alkoxyl radicals,
iv) decomposing peroxides by converting them to non-radical products, such as alcohols, and chain breaking whereby intermediate radicals such as peroxyl and alkoxyl radicals are scavenged to prevent continued hydrogen abstraction.

(Taken from Aruoma, 1994) .

1.5.1 Lipid peroxidation

Lipid peroxidation is most usually taken to mean the oxidative destruction of polyunsaturated fatty acids (PUFAs), in an autocatalytic uncontrolled process leading to the formation of fatty acid hydroperoxides and to secondary products including a wide range of aldehyde compounds.

Biological membranes and lipoproteins are susceptible to lipid peroxidation because they contain the substrates for this damaging process. The less saturated a fatty acid, the more susceptible it is to oxidation.

Lipid peroxidation is classically considered to proceed in three phases:

i) initiation,

ii) propagation

iii) termination

1.5.1.1 Initiation

Initiation is the abstraction of a hydrogen atom from the target fatty acid (LH) to form a lipid (fatty acid) radical (L·). The initiating radical could be any radical species sufficiently oxidising to react with a PUFA, thus the hydroxyl radical, most peroxyl radicals and most alkoxyl radicals are capable of starting the reaction. The superoxide radical however is not sufficiently oxidising to react with fatty acids. Certain iron complexes are capable of initiating lipid peroxidation and are widely used *in vitro* (Minotti and Aust, 1987).

The susceptible target of this hydrogen abstraction reaction is a methylene group with a double bond on either side of it (a bis-allylic methylene group). For this reason it is necessary that there are two or more bonds for a fatty acid to be prone to oxidation. The product of this reaction is a fatty acid radical that rapidly rearranges to form a conjugated diene structure. In aerobic systems, the radical reacts rapidly with oxygen to form a lipid (fatty acid) peroxyl radical (LOO·). Lipid peroxyl radicals are capable of reacting with other PUFAs and beginning a new chain of oxidation, thus forming a lipid hydroperoxide (LOOH) on the original PUFA and generating a new fatty acid radical. The lipid hydroperoxide is generally considered as the primary product of lipid peroxidation. It retains the conjugated diene structure which gives it a characteristic UV absorption allowing it to readily detected (Corongiu *et al.*, 1986).

1.5.1.2 Propagation

Propagation is the initiation of a new chain by a lipid peroxyl radical. Lipid peroxidation is an autocatalytic chain reaction, having the potential to consume all the available PUFA in a system once initiated. Although chain termination reactions usually arrest the extreme damaging potential of lipid peroxidation.

Because of the bond rearrangement reactions that occur immediately after hydrogen abstraction, different lipid hydroperoxide isomers can be formed. Hence the more double bonds there are the more positional isomers that can be produced. Unsaturated lipid hydroperoxides are prone to further oxidation, generating many secondary monomeric and polymeric oxidation products (Frankel, 1987).

Lipid hydroperoxides are not very stable. Ferrous and ferric salts react rapidly with lipid hydroperoxides to generate lipid alkoxyl radicals and lipid peroxyl radicals respectively:

$$LOOH + Fe^{2+} \rightarrow LO^{\cdot} + OH^{-} + Fe^{3+}$$

$$LOOH + Fe^{3+} \rightarrow LOO^{\cdot} + H^{+} + Fe^{2+}$$

The presence of metal ions results in loss of lipid hydroperoxides with the production of free radical species. These free radicals are subsequently capable of initiating new chains of lipid peroxidation. Ferrous salts are more soluble, react more rapidly with peroxides and generate very reactive alkoxyl radicals whereas ferric salts generated the less reactive peroxyl radicals. Therefore the complexation of iron by chelating agents does not always prevent such reactions and in some cases may promote the reaction by keeping iron in solution. EDTA can form an iron complex that promotes lipid peroxidation if used at the appropriate metal:chelator ratio (Minotti and Aust, 1987). Desferrioxamine, however, inhibits the reaction (Gutteridge *et al.*, 1979).

1.5.1.3 Termination

The lipid peroxidation reaction is substrate limited. Radical-radical reactions also occur when the conditions are favourable and these also have the effect of terminating the chains of lipid peroxidation since non-radical products are produced:

$$L^{\cdot} + L^{\cdot} \rightarrow \text{non-radical products}$$

$$LOO^{\cdot} + L^{\cdot} \rightarrow \text{non-radical products}$$

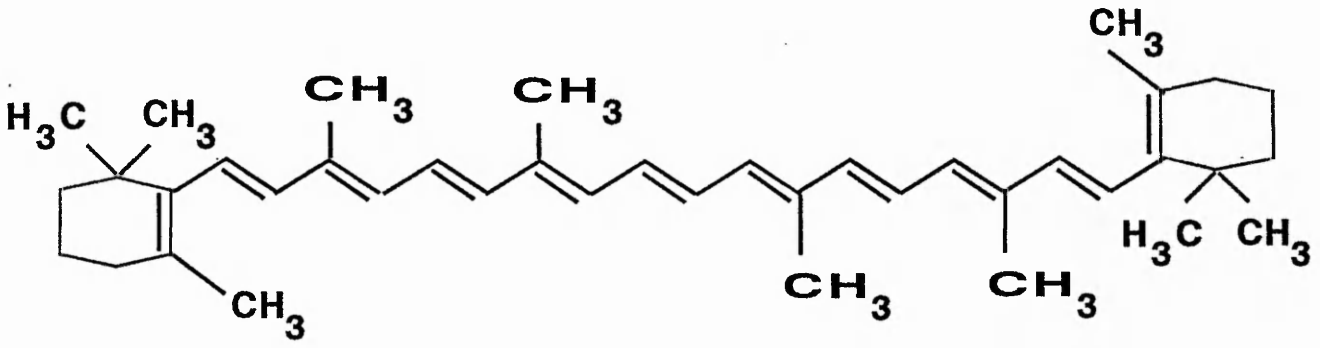
These radical-radical reactions require the individual reactants to be present at relatively high concentrations to make their interaction feasible. The reaction of two lipid peroxyl radicals may also yield reactive singlet oxygen.

1.5.2 Lipid peroxidation products

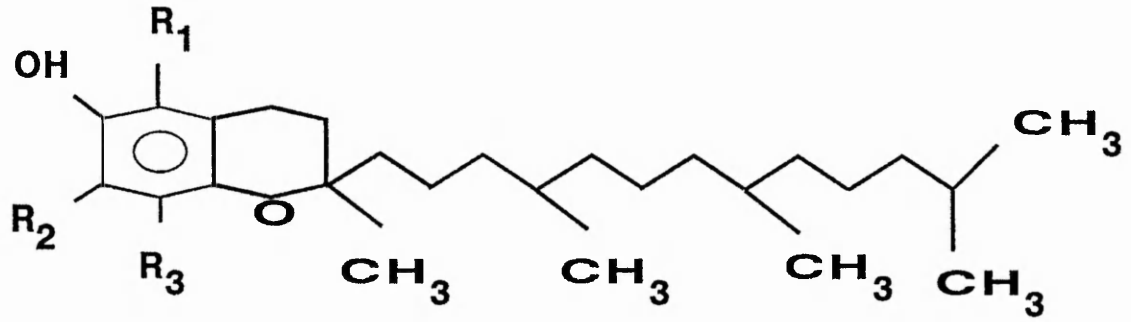
A product released from peroxidation of lipids, *trans*-4-hydroxy-2-nonenal, has been shown to form etheno-bridged DNA bases (Bartsch *et al.* 1994). Ethenonucleotides in DNA lead to misincorporation of bases upon replication or transcriptions. The formation of 1,N-etheno-(2'-deoxy)adenosine and 3,N-etheno(2'-deoxy)cytidine *in vitro* from a rat liver microsomal suspension in the presence of lipid peroxidation-inducing agents and nucleotides has been shown to occur.

1.5.3 Vitamin A

In nature vitamin A is found largely as an ester, and consequently is highly soluble in organic solvents but not in aqueous solutions. The term vitamin A is employed generically for all derivatives of β-ionone (other than carotenoids) that possess the biological activity of trans retinol or are closely related to it structurally. The parent substance of vitamin A group is called all-trans retinol, the structure of which is given in Figure 1.11. The term provitamin A is used as a generic indicator for all carotenoids that show the biological activity of vitamin A. The most active and quantitatively



ß-Carotene



Tocopherols

-Tocopherol = R_1, R_2, R_3, all CH_3

-Tocopherol = R_1, R_2, CH_3; R_3, H

-Tocopherol = R_1, R_3, CH_2; R_3, H

-Tocopherol = R_1, CH_3; R_2, R_3, H

Figure 1.11 Antioxidant inhibitors of peroxidants

the most important of these provitamins is all-trans β-carotene (Figure 1.11). Generally carotenoids must contain at least one β-ionone ring that is not hydroxylated in order to show vitamin A activity.

β-Carotene is the essential precursor to retinol or vitamin A. Since carotenes are the major precursor forms occurring naturally in fruit and vegetables this provitamin appears important in accounting for part of the protective action of fruits and vegetables. Carotenes are also excellent antioxidants and radical trapping agents, especially for peroxyl and hydroxyl radicals. The latter have been postulated to have been involved in the genesis of a number of cancers and also contribute to the ageing process.

Vitamin A is very susceptible to peroxidation. As an effective antioxidant, vitamin E might be expected to protect vitamin A. Indeed, vitamin E-deficient animals absorb and store vitamin A more poorly and deplete existing liver reserves of vitamin A more rapidly than do normal animals (Machlin, 1984). High levels of dietary vitamin A increase the vitamin E requirement. Many effects of vitamin A toxicity can be counteracted by vitamin E administration in animals.

1.5.4 Vitamin E

Vitamin E consists of four α-tocopherols and four tocotrienols, which are chain-breaking antioxidants. α-Tocopherol (Figure 1.11) is *in vivo* the most abundant and the most bioactive tocopherol. The tocotrienols differ from the tocopherols in that they have an unsaturated sidechain. The chroman head group is said to be responsible for its antioxidant activity (Burton *et al.*, 1980), while the phytyl sidechain retains the molecule in the membrane. In lipid bilayers α-tocopherol is oriented with the chroman head group towards the surface and with the sidechain buried in the hydrocarbon region. The aromatic ring lies in the proximity of the carbonyl groups of the fatty acid chains, and the phenolic hydroxyl group lies near the surface region of the bilayer. It has been hypothesised that $LOO^{\cdot}$, which has a large dipole moment, is hydrophilic and floats towards the bilayer surface. α-Tocopherol may meet it halfway by moving up and down around an average position (Burton *et al.*, 1986).

The chain breaking activity of α-tocopherol is well understood. The tocopherol molecule (TOC-OH) intercepts a peroxyl radical and is converted to a free radical form (a phenoxyl radical, TOC-O·):

$$LOO^{\cdot} + \text{TOC-OH} \rightarrow LOOH + \text{TOC-O}^{\cdot}$$

The tocopherol radical can also terminate another chain by intercepting another peroxyl radical but is irreversibly transformed. In cells it is thought that tocopherol may be regenerated by reaction with a reducing agent such as ascorbate or glutathione. α-Tocopherol is present in cell membranes in proportions of one tocopherol molecule to several hundred PUFA molecules and yet it is a very effective antioxidant. The features that make α-tocopherol so effective are:

i) its combination of chromanol head (the radical scavenging end) with lipid soluble tail (to locate it in the membrane),
ii) the stability of its radical form, preventing it initiating lipid peroxidation itself,
iii) the ability to regenerate the parent molecule from the radical.

King and McCay (1983) proposed that superoxide interacts with hydrogen ions to produce hydrogen peroxide, which is then distributed in both the aqueous and membrane phase of the cell. Glutathione peroxidase, a selenium-containing enzyme, destroys the hydrogen peroxide in the aqueous phase, thus shifting most of the hydrogen peroxide into the membrane. Any hydrogen peroxide remaining in the membrane may react with superoxide anion to form hydroxyl radicals which can react with tocopherol localised in the membrane. If insufficient tocopherol is available to trap OH', this extremely reactive radical may initiate peroxidation of PUFA in the membrane. A scheme summarising some of these concepts is given in Figure 1.12.

Vitamin E has been shown to inhibit chemically induced cancers in rats, mice and hamsters. It has been postulated that such antioxidants may reduce the activation of various carcinogens to epoxides, which are more effective than the parent compounds in producing malignant transformation (Burton *et al.*, 1986). Vitamin E inhibits both *in vitro* and *in vivo* formation of nitrosamines, compounds that are generally carcinogenic (Burton *et al.*, 1986). α-Tocopherol

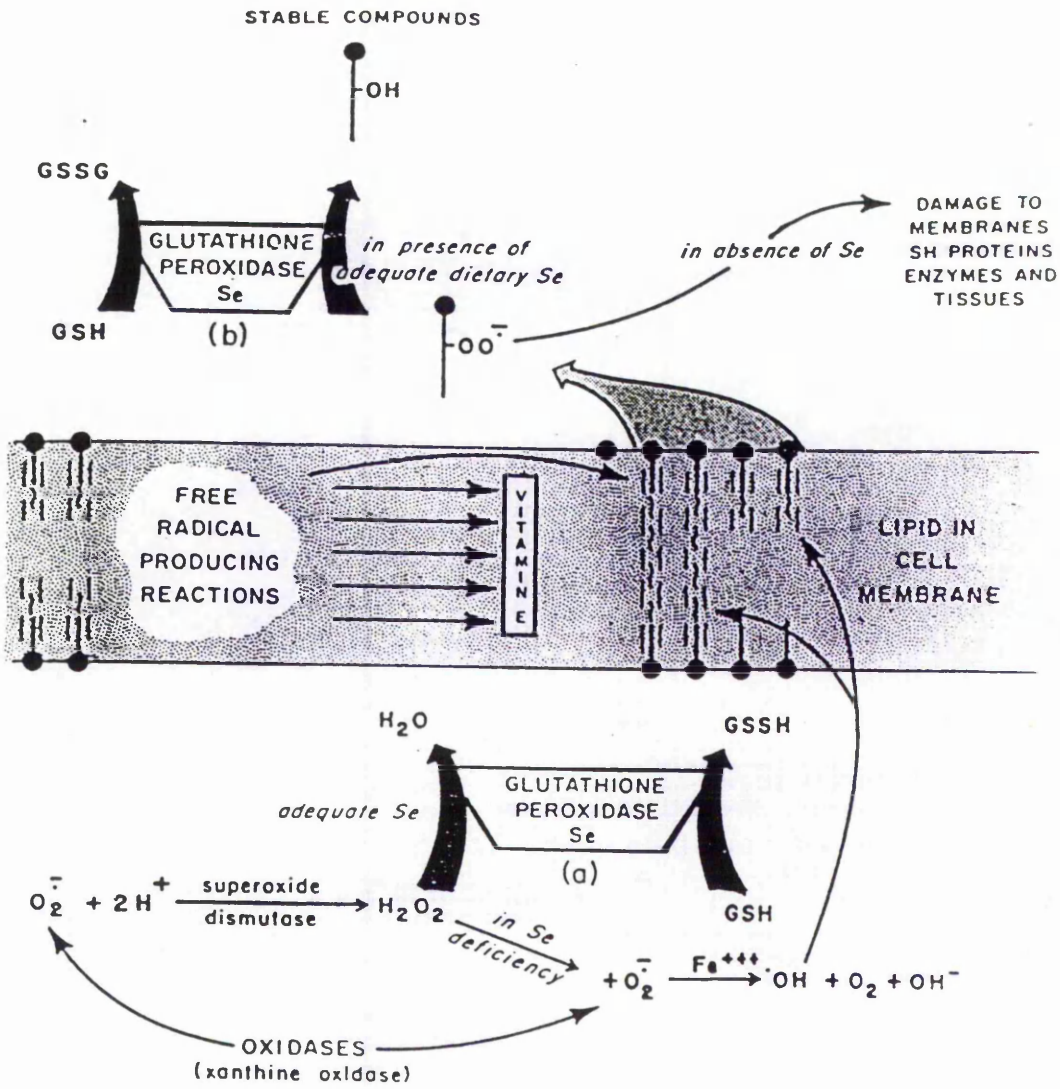


Figure 1.12 Interrelationships of glutathione peroxidase and vitamin E in protecting membranes
(Modified from Machlin, Handbook of Vitamins, Marcel Dekker Inc., New York and Basel, 1984.)

prevents nitrosamine formation by irreversibly reacting with nitrosating agents to form α-tocopheryl quinone. The presence of α-tocopherol in bacon remarkably reduces nitrosamine formation during frying.

There is *in vitro* evidence that vitamins E and C are synergistic in their antioxidant properties (Machlin, 1984). Furthermore, *in vitro* ascorbate can reduce the tocopherol phenoxy radical with the concurrent formation of an ascorbate radical (Bendich *et al.,* 1982). The ascorbate radical can be enzymically reduced back to ascorbate by an NADH-dependent system. Evidence for this mechanism operating *in vivo* is lacking and although ascorbate clearly spares selenium (Machlin, 1984), there is no definite evidence of a sparing effect on vitamin E.

1.5.5 Vitamin C

Vitamin C, or L-ascorbic acid, is a water soluble vitamin widely distributed in nature. Ascorbic acid consists of a lactone ring that ionises by hydrogen ion release at the hydroxyl C_2 and C_3 positions to give dehydroascorbate, forming a redox system (Figure 1.13). The ability of ascorbic acid to reduce iron *in vivo* is important in free radical reactions.

It is believed that ascorbate is able to regenerate vitamin E. Since ascorbate is water-soluble the potential for ascorbate to regenerate α-tocopherol would be in the outer region of the cell membrane where the chromanol head is located.

The exact mechanism in which ascorbate works to inhibit lipid peroxidation is uncertain. Ascorbate radicals were shown to increase while α-tocopherol radicals decreased during pulse radiolysis of an aerated tocopherol solution (Packer *et al.*, 1979). There was an increase in the phenoxy radical formation, which subsequently disappeared as the ascorbate radical increased. It was proposed that the ascorbate radical 'semidehydroascorbic acid' was enzymically reduced back to ascorbic acid by a nicotinamide adenine dinucleotide reaction system (Schneider and Staudinger, 1965). This mechanism would then enable continuous regeneration of the α-tocopherol to enable antioxidant function to continue.

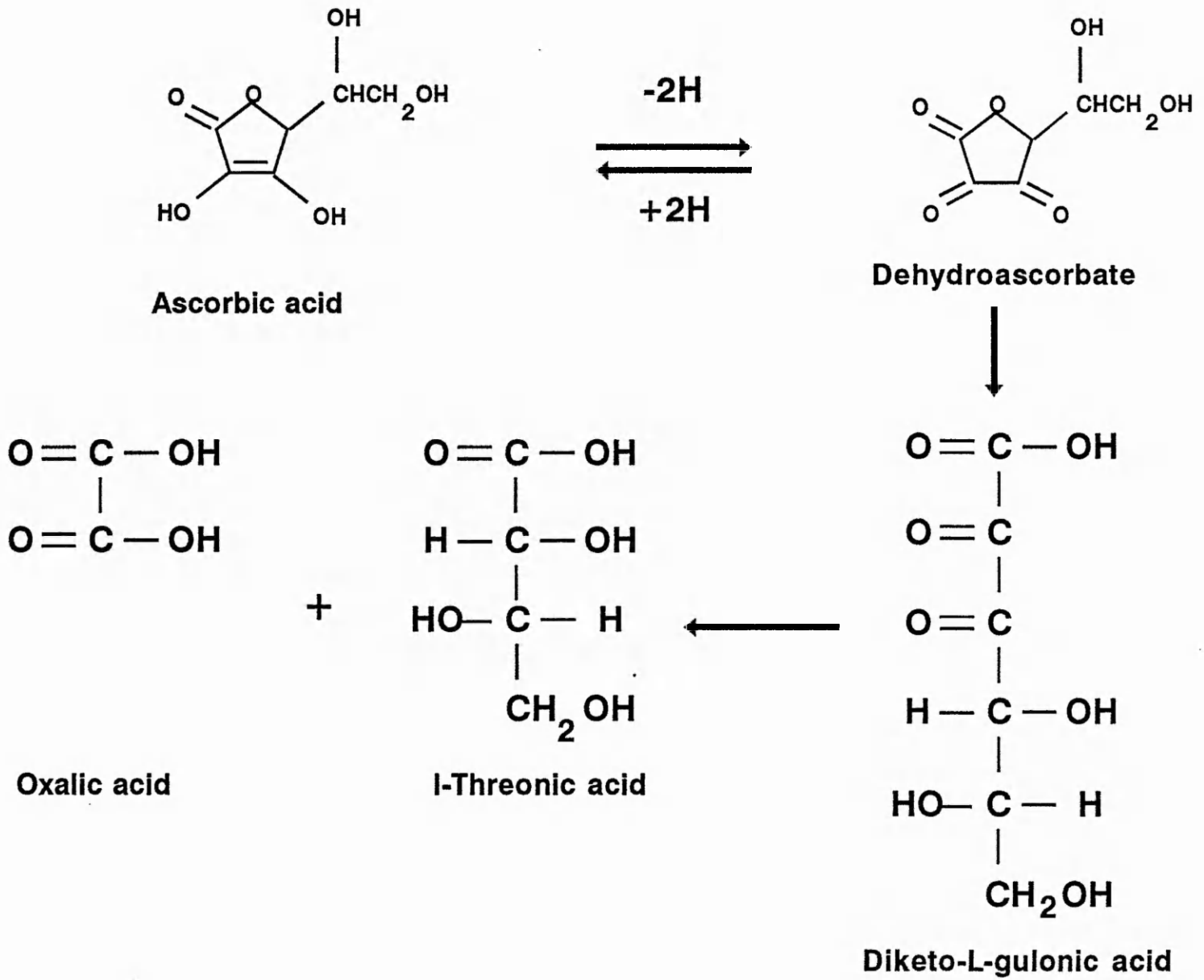


Figure 1.13 Structure of ascorbic acid and its oxidised forms

The possible role of vitamin C in cancer prevention, therapy and management is under intensive study. Vitamin C has been related to immunostimulation and deficiencies in tissue where its activity as an antioxidant and/or interaction with free radicals is impaired (Cameron, 1982). The formation of nitrosamines and other N-nitroso compounds, known genotoxins and/or carcinogens, derived from foods, metabolic processes, smoking and environmental sources, is prevented by L-ascorbic acid, however the vitamin has no anti-carcinogenic effect with preformed nitrosamines and related substances (Schmahl and Eisenbrand, 1982). Large supplements of vitamin C were reported to improve the quality and length of life of cancer patients and to cause remissions.

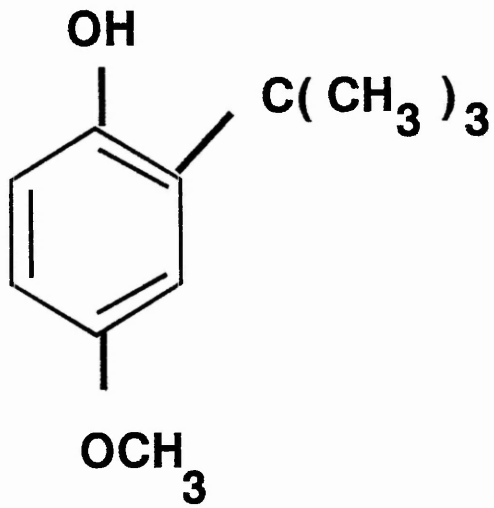
1.5.6 Butylated hydroxyanisole and butylated hydroxytoluene

The phenolic compounds 'butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT)' are widely used as antioxidants in the food industry as well as in chemical and pharmaceutical preparations, for their activity as free radical scavengers (Figure 1.14). They act by a chain breaking mechanism similar to that of vitamin E. They efficiently donate a hydrogen atom to a peroxy or alkoxy radical, so interfering with the propagation of lipid peroxidation:-

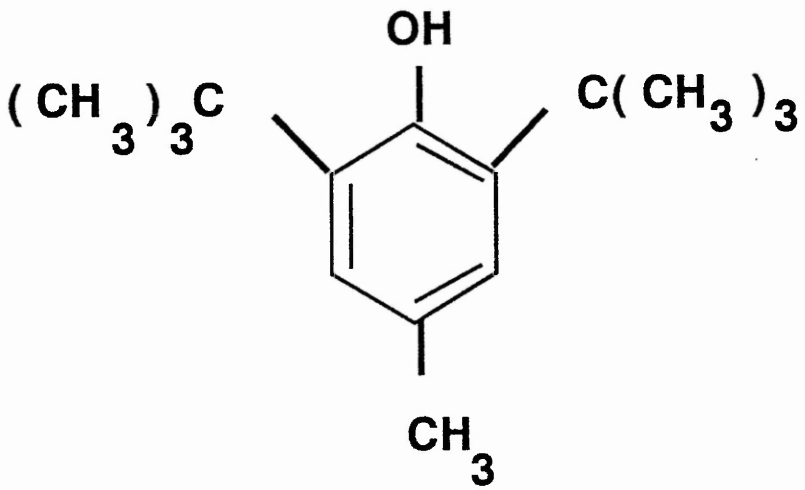
$$A\text{-}OH + RO_2^{\cdot}(RO^{\cdot}) \longrightarrow A\text{-}O^{\cdot} + RO_2H(ROH)$$

$$A\text{-}NH + RO_2^{\cdot}(RO^{\cdot}) \longrightarrow A\text{-}N^{\cdot} + RO_2H(ROH)$$

The human consumption of synthetic antioxidants is in the order of 10 mg/day (Halliwell and gutteridge, 1984). They act as chain breaking antioxidants, intercepting peroxyl radicals before they react further with unsaturated lipid molecules. In some instances there is evidence that BHA and BHT will prolong life and inhibit chemically induced carcinogenesis (Laughton *et al.*, 1989). There is complementary evidence that DNA binding of the carcinogen is reduced by these dietary additives. Although high dose rates of these phenolic compounds have resulted in cancer producing effects. Phenolic antioxidants can stimulate free radical damage to non-lipid components such as DNA, proteins and carbohydrates, *in vitro* (Laughton *et al.*, 1989). Thus when evaluating an antioxidant the following must be taken into consideration:



Butylated hydroxyanisole



Butylated hydroxytoluene

Figure 1.14 Structure of BHA and BHT

i) Can the antioxidant cause damage in biological systems different from those in which it exerts protection,

ii) is the antioxidant protection the primary biological role of the molecule or a secondary one,

iii) if the antioxidant acts by scavenging reactive oxygen species (ROS) can the antioxidant derived radicals themselves do biological damage.

Synthetic antioxidants induce many of the enzymes involved in the detoxication of carcinogenic intermediates. Hepatic glutathione transferase activity in both mice and rats is induced by a number of phenolic antioxidants, dietary BHA being particularly effective and this results in increased induction of up to 9-fold (Rahimtula *et al.*, 1982). This induction by BHA is the result of increased synthesis of the enzyme. GSTs are a family of cytosolic enzymes with differing substrate specificities and the level of induction depends to some extent on the substrate. There is also a microsomal form of GST which was found to be less susceptible to induction by BHA.

Dietary BHA and BHT elevate the activity of UDP-glucuronyl transferase in the mouse and rat liver (Rahimutala *et al.*, 1982). This enzyme utilises UDP-glucuronic acid (UDPGA) which is maintained at low concentrations in the cell and is produced by a NAD-requiring dehydrogenase which is also stimulated by dietary BHA and BHT. These compounds also elevate the activity of quinone reductase (ST-diaphorase) which would, in the presence quinones, generate NAD from NADH and therefore stimulate the production of UDPGA. The stimulation of DT-diaphorase by phenolic antioxidants may also provide increased protection against the carcinogenic effects of quinones as this enzyme catalyses the 2-electron reduction of quinones to metabolites which cannot redox cycle and form mutagenic oxygen radicals (Chesis *et al.*, 1984).

Thus synthetic dietary antioxidants increase the capacity of cells in a variety of organs to conjugate many of the intermediates formed during metabolism of carcinogens. This is probably a spin off from BHA and BHT excretion as glucuronide conjugates or mercapturic acid derivatives (metabolites of GSH conjugates) and the increase in activity of these conjugating enzymes is probably designed to accelerate elimination of the antioxidants themselves (Gower, 1988).

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 CHEMICALS

All chemicals used were of the highest grade available. Unless otherwise stated, these were purchased from either Sigma Chemical Co., Poole, Dorset (UK) or Fisons plc, Loughborough (UK). Sunset Yellow (C.I. 15985) and carmoisine (C.I. 14720) were obtained from BDH Diagnostic Division, Gurr. It should be noted that purity of the azo dyes was 85%. Each azo compound and aromatic amine is listed with its colour index (C.I.) in Table 2.1. Their structures are shown in Figure 1.1.

Bacteriological agar and nutrient broth no.2 were obtained from Gibco Ltd., Paisley, Scotland (UK). Whitley Impedance Broth (WIB) was obtained from Don Whitley Scientific Ltd., Shipley, UK. All other bacteriological media was purchased from Oxoid, Basingstoke, UK.

1-Amino-2-naphthol-6-sulphonate and 1-amino-2-naphthol-3,6-disulphonate were generous gifts from Dr. I. Coutts, Department of Chemistry and Physics, The Nottingham Trent University, UK. 2-Deoxyguanosine (2dG) and 8-hydroxy-deoxyguanosine (8-OHdG) were obtained from Dr. J.K. Chipman, School of Biochemistry, Birmingham University.

2.2 BACTERIAL STRAINS

The organisms studied were obtained from The Nottingham Trent University Culture Collection, except *Salmonella typhimurium* strains, which were obtained from Prof. B. Ames, University of California, USA.

TABLE 2.1 Colour Index of azo compounds

Azo Compound	Alternative Name	Colour Index Number
Azo-benzene	?	
p-Phenylazophenol	Solvent Yellow 7	11800
Sudan 1	Solvent Yellow 14	12055
Orange G	Food Orange 4	16230
Orange II	?	15510
Orange I	Acid Orange	14600
Sunset Yellow	FD&C Yellow 6	15985
Bordeau Red	Acid Red 17	16180
Amaranth	FD&C Red 2	16185
Ponceau 6R	Food Red 8	16290
Erriochrome Blue Black	Mordant Black 3	14640
Carmoisine	Food Red 3	14720
Sudan III	Solvent Red 23	26100
Ponceau S	?	27195

2.2.1 Preparation of bacterial cell suspension

Enterococcus faecalis was grown in tryptone soya broth, *Bacteroides thetaiotaomicron* was grown anaerobically (Compact Anaerobic Cabinet, Don Whitley Scientific Ltd., Shipley, UK) in Schaedlers broth, both were incubated at 37°C for 24 h. Cells were harvested by centrifugation at 7890xg for 10 min and anaerobically washed thrice with 0.5 M potassium phosphate buffer (pH 7.4). The bacterial pellets were resuspended to one twentieth of their previous volume in phosphate buffer and immediately used for azo reduction.

2.2.2 Preparation of *E. faecalis* cell extracts

Several methods were employed to lyse *E. faecalis*.

i) Sonication

Cell free extracts were prepared by sonicating whole cell suspensions for 20 min at a frequency of 20kHz and maximum power output under a continuous flow of nitrogen gas. The cell suspension was externally cooled by a salt/ice mixture. Cell debris was removed by centrifugation at 12500xg, 5°C for 30min.

ii) Enzymic

The method used was essentially that of Monsen and colleagues (1983). To 5ml of *E. faecalis* suspension was added 50µl EDTA (0.5M), 125µl Triton X-100 (20%, w/v) and 100µl M-1 muramidase (5mg/ml). After incubation at 37°C to obtain complete visual lysis, usually 5-60 min, cell debris was removed by centrifugation at 40,000xg for 30 min. The cell extract was stored at -20°C until required for use.

2.2.2.1 Protein Assay

All protein assays were carried out using the Bio-Rad protein assay kit. A standard curve was prepared using known concentrations of bovine serum albumin (stock solution: 1mg/ml). The Bio-Rad solution was diluted with distilled water (20%, w/v). This diluted sample may be stored for up to 14 days at room temperature. Before use the diluted reagent was filtered using Whatman 1 filter. To 2.5ml of Bio-Rad reagent was added 500µl of each standard

bovine serum albumin concentration and incubated at room temperature according to the manufacturer's instructions. The optical density at 595nm was read after 20 min and a calibration curve plotted of optical density against protein concentration. Unknown protein samples were similarly assayed using the Bio-Rad protein assay kit and the optical densities measured. From the standard curve the concentration of protein present in each sample was calculated.

2.3 PURIFICATION OF AZO DYES

2.3.1 Thin layer chromatography

Thin Layer Chromatography (TLC) was carried out on glass plates coated with silica gel (1mm) or cellulose (1mm). These were prepared by mixing 60g of silica or cellulose with 120ml of distilled water. The resultant slurry was spread uniformly on to the plates with the help of a spreading template (Camlab Ltd., Cambridge, UK). Prior to use the plates were activated by placing in an oven at 100°C for 15 min. The plates were equilibrated with the respective chromatographic solvent for 20min. The dye was dissolved in the minimum quantity of distilled water. The solution (10µl) was spotted onto the plate and allowed to dry. The plate was then run in a tank containing one of the following solvent systems.

i) methanol - 28% aqueous ammonia - distilled water
ii) pyridine - methanol - 28% aqueous ammonia - distilled water
iii) n-butanol - ethanol - distilled water (1:1:1)
iv) NaCl - 50% ethanol
v) Methanol - chloroform

The plates were shielded from direct sunlight by covering the tanks with aluminium foil.

The chromatograms were visualised under UV light. The pure components were well isolated from any impurities. Following extraction into methanol, samples were centrifuged in a bench centrifuge for 10 min at maximum speed to remove silica or cellulose. Methanol was

subsequently evaporated to dryness by blowing with nitrogen. The purity of the dyes was checked on HPLC. Each dye was stored in the dark at room temperature.

The Rf value of each spot was measured and the colour noted. The Rf ratio is a measure for linear chromatography. It permits correlation of data obtained from separations of the same material carried out using different solvent systems.

$$Rf = \frac{\text{Distance moved by substance from origin}}{\text{Distance moved by solvent front from origin}}$$

2.3.2 Column chromatography using chitosan

Chitosan is a porous hydrophilic compound which adsorbs a high content of azo dyes. A slurry of the dye and chitosan was prepared by shaking a mixture of amaranth and distilled water (3g/25ml) with 10g chitosan. The addition of the solution was continued until no more dye was adsorbed on the chitosan and the non-adsorbed dyes began to colour the supernatant. The slurry was poured into the column and the system was allowed to equilibrate. The column was eluted with hot water and acetone. The dye should remain adsorbed to the chitosan. The dye is eluted with methanolic sodium hydroxide (1 g NaOH in 70% methanol). The pH of the eluate was adjusted to 5-6 using methanol acetic acid (1:1). This eluate is mixed with fresh chitosan and the procedure repeated. Finally the eluate is treated with acetic acid and evaporated to dryness. Purity was checked by TLC and uv/vis spectrophotometry.

2.3.3 Cellulose flash chromatography

Cellulose was pre-soaked in the eluent (water:butanol:ethanol) for 5 min. A 1cm (i.d) flash chromatography column was filled with the swollen cellulose such that the column bed was 20-30cm when packed. Air pressure was applied to pack the column. The column was flushed with 100ml of eluent to remove any air bubbles in the bedding. The dye (1g) to be tested was dissolved in sufficient eluent and applied to the column. The column was then filled with

100ml of eluent and light air pressure was applied to increase the flow through the column to 5.5ml/min (the level of solvent dropped approximately 8cm/min). All effluents were monitored by TLC as described previously and final purity was confirmed by HPLC.

2.3.4 High Performance Liquid Chromatography

A reverse-phase high-performance liquid chromatography (HPLC) system was developed for analysis of the azo dyes. A model 7125 system was used in conjunction with a UV detector. Purity checks were carried out at a flow rate of 1ml/min on a 30 x 0.5cm μbondapak C_{18} reverse phase column with a Guard-Pak precolumn inserted. The columns were from Waters Associates, Milford (UK). The mobile phase was a linear gradient of 20-80% methanol in ultra pure water over 60min.

2.4 AZO REDUCTION ASSAYS

2.4.1 Bacterial reduction of azo dyes

The standard assay mix consisted of 4.2 ml degassed potassium phosphate buffer (50 mM, pH 7.4), 0.4 ml washed cell suspension, with or without glucose (0.2 ml, 10%w/v) and electron carriers in narrow assay tubes (18 x 142 mm). The various electron carriers were prepared anaerobically at 0.025 mM (final concentration). The reaction mix was incubated at 37°C prior to starting the reaction by the addition of 0.1 ml of the appropriate azo dye (10 mM). Decolouration rate was determined spectrophotometrically at the wavelength of the maximal absorbance of the azo dye.

2.4.1.1 Culture age and its effect on azoreductase activity of *E. faecalis*

A 5% inoculum of an overnight culture of *E. faecalis* was added to Todd Hewitt Broth. This was incubated at 37°C. Samples (10ml) were removed at 1, 2, 3, 4, 5, 7, 8, 24 and 26 hour intervals after inoculation. The samples were washed in potassium phosphate buffer (50 mM, pH 7.4) and finally resuspended in 5ml of the same buffer. The optical density of the samples was measured at 650nm.

The azoreductase assay was essentially as described above with the following modifications: 1ml cell suspension, 200µl azo dye (1mM), 100µl glucose (10% w/v) and 1.7ml potassium phosphate buffer (50mM, pH 7.4). Decolouration rate was determined spectrophotometrically at the wavelength of the maximal absorbance of the azo dye.

2.4.1.2 Overlay method for detecting azoreductase activity

A plate assay was developed for the detection of bacteria that exhibit azoreductase activity. An overnight culture (100µl) diluted 10^{-5} to 10^{-9}, was plated on bacteriological agar plates containing glucose (10%w/v) and azo dye (final concentration 1mM). The plates were

incubated under both aerobic and anaerobic conditions at 37°C and observed for clearance of the dye surrounding the colonies.

2.4.2 Chemical Reduction of azo dyes

2.4.2.1 Reduction of azo dyes by sodium dithionite

Dithionite reductions were performed by mixing freshly prepared sodium dithionite with azo dye dissolved in 0.1M potassium phosphate buffer, pH 7.5, containing 0.5% w/v β-cyclodextrin. The method followed was essentially that of Zbaida and Levine (1992). A molar ratio of 4:1 sodium dithionite: azo dye was essential for obtaining a significant reduction rate at room temperature.

2.4.2.2 Reduction of azo dyes by stannous chloride

The method used was that of Gasparic (1977). Stannous chloride (1g) was dissolved in 4ml concentrated HCl. The solution was kept at 37°C and was only used when all the stannous chloride had dissolved.

2.5 GENOTOXICITY TESTING

2.5.1 Rapid Automated Bacterial Impedance Technique (RABIT)

Prior to using the RABIT to estimate numbers of bacteria a calibration curve was generated (appendix 1). This curve defines the relationship between impedance and bacterial viability as determined by the standard plate count method.

Calibration curves were obtained for all bacterial strains monitored using the RABIT. A 10% inoculum of an overnight culture of each strain was prepared in tryptone soya broth. This was incubated at 37°C for 1 h, except in the case of *E. coli* CM871 which was incubated for 2h. A range of serial dilutions were prepared of which 300µl was transferred to a conductance cell containing 2ml of WIB and 100µl was spread on a TSB plate. The conductance cell was then placed in the incubation unit set at 37°C. The spread plates were inverted and incubated for 24 h at 37°C, after which time colonies were counted manually. The calibration curve covered a five log cycle range.

Colony forming units obtained from plate counts were compared with the measured detection time for a given sample. The RABIT software enables the plate count data to be entered into the computer to produce a plot of log CFU/ml versus Impedance Detection Time.

2.5.2 Differential Genotoxicity Assay

The *E. coli* differential genotoxicity assay was used to determine the genotoxic activity of the azo dyes based on a method originally described by Tweats *et al.*(1981), as modified by Forsythe (1990) for impedance microbiology. The tester strains were *E. coli* WP2, *E. coli* AB1157 (repair proficient) and *E. coli* CM871, *E. coli* BH20 (repair deficient). *E. coli* CM871 is a *uvr*A *rec*A *lex*A triple mutant that combines extreme repair deficiency with near wild-type growth. *E. coli* BH20 is isogenic with *E. coli* AB1157. It exhibits very low if any fapy-DNA glycosylase activity in crude lysates as compared with *E. coli* AB1157 (WT).

Compounds were accepted as genotoxic if survival of the repair deficient strain was at least four fold lower than survival of the repair proficient strain (See section 2.5.2.9).

All compounds were tested for genotoxicity with and without prior incubation with either *E. faecalis* or *B. thetaiotaomicron* cell suspensions. Incubation was for one hour with *E. faecalis* or overnight with *B. thetaiotaomicron.* Compounds were tested over a range of concentrations up to their limit of solubility or up to concentrations that caused greater than two log reduction in cell numbers of the wild type. Azo dyes were tested in the absence and presence of S9.

Genotoxic activity was assessed after removing bacterial cells by centrifugation in a microfuge (8,000xg, 5 min), followed by filtration (0.2 µm Dynaguard filters, New Brunswick). An aliquot (100µl) of the filtrate was added to the diluted *E. coli* tester strain (10^7 CFU/ml 200µl). Following pre-incubation at 37°C for 30min, the test components were mixed by vortexing and 300µl was delivered to 2ml of WIB in a conductance cell. These are subsequently loaded into the RABIT module and incubated for 24h at 37°C. The genotoxic activity was subsequently determined from *E. coli* survival number. This assay was carried out under both aerobic and strict anaerobic conditions. Inhibition by UV was used as a positive control for CM871.

2.5.2.1 Effect of continued incubation of azo dyes with *E. faecalis* and *B. thetaiotaomicron* on genotoxicity

Azo dyes were incubated with washed cell suspensions of either *E. faecalis* or *B. thetaiotaomicron* over a period of 24 hours. Samples removed at known time intervals were assessed for genotoxicity.

2.5.2.2 Stability of azo dyes after azo fission

After filtration of the reduced dye, the filtrate was incubated at 37°C. Samples were removed at known time intervals and assessed for genotoxicity.

2.5.2.3 Genotoxicity of azo dyes after chemical azo reduction

Azo dyes (5mM) were reduced by both sodium dithionite and stannous chloride methods as described previously (Section 2.4.2). The reduced dye was assessed for genotoxicity using the differential genotoxicity assay as described. Sodium dithionite was added at 0.05mg/ml as high concentrations of dithionite were too toxic to cells.

2.5.2.4 S9 Activation

Standard Aroclor-induced rat liver S9 supernatant fraction was obtained from Sprague-Dawley rats by the procedure of Ames *et al.* (1975). There is evidence that optimal concentrations of S9 fraction tend to be lower with liquid incubation than in a plate incorporation assay such as the Ames test (Forster *et al.*, 1980; Kuroki *et al.*, 1979). Therefore the differential killing test as described was extended to include a treatment with one-fifth the amount of S9 in the mix.

2.5.2.5 Differential genotoxicity assay on commercial end-products

The *E. coli* differential genotoxicity assay was used to determine the genotoxic activity of the commercial metabolites of azo dyes. Compounds were tested over a range of concentrations up to their limit of solubility or up to concentrations that caused a greater than two log reduction in cell numbers of the wild type *E. coli* strain.

2.5.2.6 Stability of commercial azo dye metabolites

The following metabolites were tested for genotoxicity with and without incubation with *E. faecalis* and *B. thetaiotaomicron* cell suspensions: 1-amino-2-naphthol, 1-amino-2-naphthol-4-sulphonate, 1-amino-2-naphthol-6-sulphonate, 1-amino-2-naphthol-3,6-disulphonate, 1-amino-naphthalene-4-sulphonate, sulphanilic acid. Samples were tested using the differential genotoxicity assay.

2.5.2.7 Effect of 8 dietary compounds on genotoxicity of azo dyes

Before testing for the effect of various antioxidants on genotoxicity of azo dyes, the effect of these compounds alone on survival of *E. coli* was determined. Each compound was tested over a range of concentrations up to its limit of solubility.

Based on the results from these control experiments, several concentrations of each compound were selected to test in combination with the azo dye. To test for the effect of various chemical compounds on genotoxicity of both reduced and unreduced dyes, 100µl of antioxidant was added to the test solution both prior to and on incubation with *E. coli.*

2.5.2.8 Genotoxicity of superoxide anions in the differential genotoxicity assay

Superoxide anions were generated using the xanthine/ xanthine oxidase system of McCord (1977). Xanthine oxidase (Sigma 1, 10^{-2} units/ml buffer) was added to a solution of potassium phosphate buffer (50mM, pH7.8), 0.1mM EDTA, 5mmol xanthine. To confirm the presence of superoxide anions, a few crystals of NBT were added to 1ml of the solution (see section 2.7.1).

This solution (100µl) was added to 200µl of the diluted *E. coli* tester strain and the genotoxic activity determined. Superoxide dismutase was used as a control to remove superoxide radicals.

2.5.2.9 Evaluation and statistical analysis

Conclusions were based on consistent results in independent experiments. The results were analysed statistically using the method of Tweats *et al.* (1981). The individual counts were transformed using the function t=sqrt(A+0.375), where A is the individual count and t is the transformed count. The transformed figures of the treated samples were subsequently expressed as a percentage of the average of the transformed values of the untreated samples. This was then used to construct log-dose response curves for each sample.

The results were further expressed as coefficients of survival (CS),

$$= \frac{\%\ \text{survivors of repair deficient strain}}{\%\ \text{survivors of repair proficient strain}}$$

(Rosenkranz and Leifer, 1980). CS values below 1.0 indicate preferential killing of the repair deficient strain. Within the limits of the assay, survival indices below 0.30 are taken as positive, values between 0.31 and 0.95 are weakly positive and values greater than 0.96 as negative. Compounds were deemed cytotoxic where there was excessive kill (2-fold reduction in cell numbers) of the wild type *E. coli* strain.

2.6 *SALMONELLA TYPHIMURIUM* PRE-INCUBATION MUTAGENICITY ASSAY

2.6.1 Bacterial strains

The strains of *Salmonella typhimurium* used were TA98, TA100, TA102 and TA104. These were obtained from Professor B.N. Ames, Biochemistry Department, University of California, Berkeley, USA.

2.6.1.1 Preparation of permanent master cultures for long term freezing

'Permanent' master cultures of bacterial strains were stored in a -80°C freezer. Nutrient broth was inoculated from a single colony taken from a master plate. Following incubation at 37°C on a rotary shaker for 24 h spectroscopic grade dimethylsulphoxide (DMSO) was added to give a final concentration of 8.25% (v/v). These were subsequently stored as aliquots of 2ml in Nunc tubes and stored at -80°C. Permanent frozen cultures can be stored for at least two years without loss of viability provided that individual cultures which have once been thawed or opened are not returned to the freezer.

2.6.1.2 Preparation of master plates from frozen permanent cultures

Every month a master plate was prepared from frozen permanents which had been prepared as above (Maron and Ames, 1983). A loopful of bacterial culture was taken from a thawed frozen permanent culture to inoculate 10ml of nutrient broth. This was incubated at 37°C for 24h. A loopful of this culture was streaked over a minimal glucose agar plate which had been supplemented with 0.1ml each of 0.5mM biotin, 0.1M L-histidine and ampicillin solution (8mg ampicillin trihydrate/ml of 0.02N NaOH). Following incubation at 37°C for 48 h, an isolated colony was taken from the plate and resuspended in 0.3ml of 0.2M sodium phosphate buffer, pH 7.4. Four parallel streaks of this bacterial suspension were made on a minimal glucose agar plate supplemented with biotin, histidine and ampicillin. The plate was incubated at 37°C for 24h and then stored inverted at 4°C wrapped in aluminium foil. Master plates for TA102 also contained tetracycline while master plates for TA104 did not contain biotin.

2.6.1.3 Procedure for growing overnight cultures

Using a sterile loop a single sweep was taken from the master plate and inoculated in nutrient broth No.2. The amount of culture required for a mutagenicity assay depended on the size of the experiment and was based on 0.1ml culture per plate. Cultures were incubated in a shaking water bath at 37°C, 120 strokes/min for 10 h.

2.6.2 Preparation of media, reagents and agar plates

The solutions were prepared according to the methods of Maron and Ames (1983) with minor modifications. All preparations were sterilised by either autoclaving for 20 min at 121°C or by filtration using a 0.2µm Dynaguard filter (New Brunswick).

2.6.2.1 Minimal glucose agar plates

To 11.29g of bacteriological agar 700ml of distilled water was added. Vogel-Bonner salts (15ml) and 40% w/v glucose (37.5ml) were added to the autoclaved agar. These salts were prepared by mixing $MgSO_4.7H_2O$ (5g), citric acid monohydrate (50g), K_2HPO_4 anhydrous (250g) and $NaHN_4HPO_4.4H_2O$ (87.5g) in 300ml of distilled water at 45°C. The salts were added in the order indicated. Each salt was allowed to dissolve completely before adding the next. The volume was adjusted to 500ml with distilled water. This was subsequently autoclaved at 121°C for 15 min. The solutions were mixed by gentle swirling. Approximately 30ml of agar was poured into 9cm plastic petri dishes (Sterilin). The agar was left to set for 30min after which the plates were dried in a plate drier. Once dried the plates were stored at 0-4°C.

2.6.2.2 Nutrient agar

Bacteriological agar (11.29g) and nutrient broth no. 2 (17.5g) were autoclaved in 700ml of distilled water. The plates were poured in the same manner as minimal glucose agar plates.

2.6.2.3 Nutrient broth

This was prepared by autoclaving 2.5g of nutrient broth no. 2 in 100ml of distilled water.

2.6.2.4 Top agar

Top agar containing 0.6% (w/v) bacteriological agar and 0.5% (w/v) NaCl in 100ml of distilled water was autoclaved immediately before use. After allowing the molten agar to cool to approximately 60°C, 10ml of a stock solution of 0.5mM L-histidine.HCl/0.5mM biotin (stored at 0-4°C) was added. The solution was mixed thoroughly by swirling. Using aseptic techniques 2ml aliquots of top agar were delivered to glass top agar tubes in a water bath at 45°C.

2.6.2.5 Other solutions

Other stock solutions prepared were: 0.2M sodium phosphate buffer (pH 7.4); distilled water; $MgCl_2$.KCl salts (0.4M $MgCl_2$ + 1.65M KCl); 0.1M $NADP^+$ and 1 M glucose 6-phosphate. the $NADP^+$ and G6P stock solutions were sterilised by filtration and stored at -20°C. All the other stock solutions were stored at 0-4°C.

2.6.2.6 Preparation of S9 mix

S9 mix was freshly prepared and kept sterile and at ice temperature at all times during its preparation and use. Rat Liver S9 used was kindly supplied by Dr. J.K. Chipman, School of Biochemistry, University of Birmingham, UK. Table 2.2 lists the stock solutions used for preparing S9 mix. The ingredients were added in the order shown such that hepatic enzymes were added to a buffered solution. The S9 was thawed just before use.

TABLE 2.2 Stock solutions for preparing S9 mix

Constituent	per 50ml
Distilled water	16.75 ml
0.2M Sodium phosphate buffer, pH 7.4	25.00 ml
0.1M $NADP^+$	2.00 ml
1M Glucose-6-phosphate	0.25 ml
$MgCl_2$.KCl salts	1.00 ml
Rat liver S9	5.00 ml (10%)

Any unused S9 was discarded.

2.6.3 Mutagenicity assays

Two different protocols were used to evaluate the mutagenic potential of azo dyes:

i) the standard Ames plate assay with pre-incubation performed directly on the sample (Ames *et al.,* 1975),

iii) the standard Ames plate assay incorporating azo reduction with FMN and pre-incubation.

Samples were tested with each of these methods using *S. typhimurium* strains TA98, TA100, TA102 and TA104. Compounds were tested over a range of concentrations up to their limit of solubility.

2.6.3.1 Standard Dose assay

The standard Ames assay was essentially that of Ames *et al.* (1975). Each assay with metabolic activation used 500µl of Aroclor induced rat liver S9. To this was added 200µl of the test compound and 100µl of bacterial culture; this was added last to avoid placing the bacteria in direct contact with the undiluted test compounds. Negative controls containing S9 mix, bacteria in 0.2 M sodium phosphate buffer (pH 7.4), but no test compound were used to measure the number of colonies that arise spontaneously for each bacterial strain. The mutagenicity of the test compounds in the absence of mammalian enzymes was assayed by substituting 500µl of 0.2 M sodium phosphate buffer (pH 7.4) for the S9 mix. The contents were gently mixed and pre-incubated for 30min at 37°C in a shaking water bath (60rev/min). The reaction was stopped by placing the tubes on ice. Molten top agar (2 ml) was added to the tubes. The test components were mixed by vortexing for approx. 3 sec. These were then poured on to a minimal glucose agar plate. As soon as the contents were poured, the plate was tilted and swirled to ensure even distribution of the molten top agar. Within 30 min of pouring , the plates were inverted and incubated at 37°C. After 48h the revertant colonies were counted manually. The number of spontaneous revertants in each experiment were subtracted from the total number of revertants in order to obtain the reversion rate induced by the test compound. The presence of a background lawn of auxotrophic bacteria on all plates was confirmed by light microscopy.

2.6.3.2 Salmonella assay with azo reduction

The azo reduction mutagenicity assay with FMN and pre-incubation was performed as described by Prival *et al.* (1988). In the azo reduction mutagenicity assay, a modified cofactor mix that contains 2mM FMN was used to facilitate azo reduction in the presence of the tester bacteria. This modified cofactor mix contained 2.8units/ml of added glucose-6-phosphate dehydrogenase, 2mM NaOH and 20mM glucose-6-phosphate (4 times the concentration used in the Ames assay). The bacteria, test chemical and metabolic activating mixture containing 150µl of rat liver S9 per plate were pre-incubated for 30min without shaking before addition of top agar. The mix is then poured onto petri plates containing minimal medium base agar.

All colony counts reported were performed manually. All data presented, except where stated, represent mean colony counts from triplicate plates of 3 separate experiments. Mutagenic activity was considered detectable or significant when the mean mutant counts were at least two times the mean of negative control counts.

2.6.3.3 Evaluation and statistical analysis

Results were analysed using the method of Ames *et al.* (1975). Routine examination of the bacterial background lawn (e.g. whether it was normal, sparse or non-existent) as a consequence of the trace of histidine added to the top agar was an important tool in determining the toxicity of the test compound. Compounds were considered mutagenic if the following criteria were met:

(a) there is a doubling of his^+ revertants over and above that of the control,

(b) there is a sizeable dose response.

2.7 DETECTION OF SUPEROXIDE ANION RADICAL

2.7.1 Nitroblue tetrazolium assay

Superoxide anion ($O_2^{\cdot-}$), production was detected by the reduction of nitroblue tetrazolium (NBT) and its inhibition by superoxide dismutase (SOD), according to the method of Oberley and Spitz (1984). The sample (100µl) to be tested was added to 0.8 ml potassium phosphate buffer (50mM, pH 7.8) containing: 0.056mM NBT, 0.1mM EDTA, 0.1mM xanthine, 0.06 % w/v Triton X100 and 0.33 mg/ml gelatin, plus or minus 100µl SOD. Immediately after mixing the absorbance at 560 nm was measured with respect to time. SOD (Sigma type I, bovine) activity was assayed using a xanthine/xanthine oxidase technique (Oberley and Spitz, 1984). SOD was inactivated by boiling for 15 min.

2.7.2 Cytochrome C assay

Superoxide anion ($O_2^{\cdot-}$), production was detected by the reduction of horse heart ferricytochrome C (Sigma type VI) and its inhibition by superoxide dismutase (SOD), according to the method of Flohe and Otting (1984). The assay was conducted at 37°C, the sample (100µl) to be tested was added to 0.8 ml potassium phosphate buffer (50 mM, pH 7.8) containing: 50-100 mM ferricytochrome C, 50-100mM NADPH, 0.1mM EDTA, plus or minus 100µl SOD. Immediately after mixing the absorbance at 550 nm was measured with respect to time. SOD activity was assayed as for NBT assay (2.7.1).

2.7.3 The generation of superoxide radicals

Superoxide anions were generated using the xanthine/xanthine oxidase system of McCord (1977). Xanthine oxidase (Sigma 1, 10^{-2} units/ml buffer) was added to a solution of potassium phosphate buffer (50mM, pH7.8), 0.1mM EDTA, 5mmol xanthine. To confirm the presence of superoxide anions, a few crystals of NBT were added to 1ml of the solution.

2.8 HYDROGEN PEROXIDE DETECTION ASSAY

Hydrogen peroxide production detection assay was based on the peroxidase-catalysed oxidation of a suitable substrate to yield a coloured product which can be subsequently detected spectrophotometrically. The assay was based on the method of Singh (1982) with slight modification. Hydrogen peroxide, a potent oxidising agent couples oxidatively with 4-aminoantipyrene and phenol to yield a quinoneimine dye with a maximum absorption at 505nm ($e = 6.4 \times 10^3$ mol^{-1} cm^{-1}).

$$2H_2O_2 + \text{4-aminoantipyrene} + \text{phenol} \rightarrow \text{chromagen} + 4H_2O$$

The reagent solution contained: 0.234g phenol, 0.1g aminoantipyrene, 2×10^{-8} M horseradish peroxidase (Sigma) and 2.5×10^{-6} M hydrogen peroxide made up to 100 ml in phosphate buffer (0.1 M, pH 6.9). A small amount of H_2O_2 in the stock reagent eliminated any interference in the assay by trace impurities in 4-aminoantipyrene. The sample (1 ml) to be tested was added to 4 ml stock reagent and made up to 10 ml with UHQ water. The change in absorbance at 505nm was measured until a constant reading was obtained. A 4 ml aliquot of the reagent solution made up to 10 ml with UHQ water served as a blank. A standard curve was prepared using known concentrations of H_2O_2. From the standard curve the probable concentration of H_2O_2 in each sample was calculated.

2.9 OXIDATIVE DAMAGE TO DNA

2.9.1 Treatment of *E. coli* cells

E. coli strains to be treated were grown in 1l of tryptone soya broth (Oxoid)at 37°C. After 24h incubation, cells were collected by centrifugation (1,000g, 10min) and resuspended in 100ml of 100mM sodium phosphate buffer (pH 7.4). This was divided in four 25ml volumes. One part was used as a control and the other three were treated with different concentrations of tester sample at 4°C for 30 min. The cells were collected by centrifugation, suspended in 40ml

of the above buffer, and collected again by centrifugation. This washing procedure was repeated twice more and then the cells were frozen at -80°C.

2.9.2 Isolation of *E. coli* DNA

Materials

TE Buffer, pH 8.0

Tris HCl	10 mM
EDTA	1 mM

Proteinase K Digestion Buffer, pH 8.0

Proteinase K	100 mM
EDTA	5 mM
Sarcosyl	0.5% (w/v)

pH adjusted with NaOH

Chloroform Reagent

Chloroform containing 2 % isoamyl alcohol (v/v)

RNase Stock solution, pH 7.5

RNase, Type XII bovine pancreas	10 mg/ml
Tris HCl	10 mM
NaCl	15 mM

Heat to 100°C for 15 min

RNase Buffer

RNase	100 mg/ml
Tris HCl	50 mM
EDTA	10 mM
NaCl	10 mM

DNA was isolated from *E. coli* cells according to the method of Fiala *et al.*(1989) with slight modifications. Cell suspensions were washed twice in phosphate buffered saline, pelleted by centrifugation and finally resuspended in TE buffer (pH 8.0, 100µl) containing lysozyme (1 mg/ml). Proteinase K digestion buffer (1ml) was added and cells were incubated at 50°C for 2h. DNA was extracted with an equal volume of phenol and subsequent phenol/chloroform/ isoamyl alcohol (1:1:1 v/v/v) and chloroform/isoamyl alcohol (1:1 v/v) (x2) extractions. Samples were gently mixed at each stage and microfuged for 1 min at high speed. Nucleic acids were precipitated by the addition of 2.5-fold the volume of ethanol and 1/10-fold the volume of 0.3 M sodium acetate and left at -20°C for at least 3 h. The DNA and RNA were pelleted by centrifugation and dried by vacuum desiccation. The pellet was dissolved in RNase buffer (400µl) and incubated at 37°C for 2h. DNA was extracted with phenol/chloroform/ isoamyl alcohol and subsequently with chloroform/isoamyl alcohol (x2) and precipitated with ethanol and sodium acetate as before. DNA was dried as before and dissolved in TE buffer (pH 8.0, 500µl) subsequent to dialysis for 24 h, changing buffer after every 4 hours.

2.9.3 Digestion of DNA samples

To a 300µl aliquot containing approximately 100 mg DNA sample was added 12µl magnesium chloride (0.3 M). Samples were place in a boiling water bath for 3 min, -20°C for 4 min and finally refrigerated for 3 min. Samples were digested to deoxynucleosides with 7µl DNase I (200 mg/ml) and endonuclease (5µl) at 37°C for 15 h. Further incubation overnight at 37°C, after addition of Tris base (1 M, pH 8.0), 5µl freshly diluted alkaline phosphatase (5 µl/45 µl UHQ H_2O) and 8µl of phosphodiesterase (1.2 mg/400 ml UHQ H_2O), provided hydrolysates that were centrifuged and supernatants were stored at 4°C prior to same day analysis by HPLC-EC at School of Biochemistry, Birmingham University.

2.9.4 Analysis of 8-OHdG with an HPLC-electrodetector system

The DNA hydrolysates were analysed using reverse phase HPLC coupled with an electrochemical detector. The hydrolysates (10µl) were injected onto a reverse phase ultratechsphere c18 column. The eluent was 5% (v/v) aqueous methanol containing 12.5mM citrate, 25mM sodium acetate, 30mM sodium hydroxide and 10mM acetic acid, pH 5.1 with flow rate 0.8ml/min. The mobile phase was filtered through a 0.45µm Durapore membrane filter (Milipore, Watford, UK) fitted onto scintered glass filter holder apparatus (Whatman). The eluent was routed through a Pye Unicam LC-UV detector for the quantitation of deoxyguanosine (dG). A model LC-4B amperometric detector (Bioanalytical Systems, Luton, Bedfordshire, UK) was used for quantitation of the electrochemically active 8-OHdG. The amperometric detector used a glassy carbon electrode at a potential pf +0.6V (determined from a hydrodynamic voltammogram), providing selectivity for 8-OHdG, measured against an Ag/AgCl/3M NaCl reference electrode. A standard curve was constructed relating concentration of 8-OHdG to electrochemical detector response. The location of peaks in the HPLC-EC elution profiles due to dG and 8-OHdG was verified by co-injection of DNA hydrolysates with standard compounds. The molar ratio of 8-OHdG to dG in each DNA sample was determined based on the peak height of authentic 8-OHdG with the EC detector and the UV absorbance at A_{254} of dG.

After analysis of 8-10 samples the column was washed with UHQ water and finally washed and stored with 80% methanol in UHQ water.

CHAPTER 3

RESULTS

RESULTS

3.1 REQUIREMENTS FOR AZO REDUCTION

Reduction of amaranth, sunset yellow and carmoisine by a washed cell suspension of *E. faecalis* and *B. thetaiotaomicron* is summarised in Table 3.1. When the substrate was incubated with either the NADH or NADPH generating system and microbial azoreductase preparation, reduction did not commence immediately due to a lag period. The length of the lag period depended upon the amount of soluble flavin added (Figure 3.1). The reduction rate was constant until the dye concentration became low, possibly when the reaction became substrate limited.

Azoreductase activity of *E. faecalis* was stimulated by exogenous glucose, FMN and RF. The rate of reduction of amaranth by a washed cell suspension of *E. faecalis* was 40.7, 113.5 and 83.6μmol/min/mg cell dry weight for added glucose, added FMN + glucose and added RF + glucose respectively (Table 3.1). The inclusion of FMN with glucose was essential for maximum activity. FAD was inferior to FMN as a cofactor in azo dye reduction, 45.6μmol/min/mg cell dry weight for reduction of sunset yellow by a washed cell suspension of *E. faecalis*. Incubation with FMN and glucose was far superior than glucose alone at stimulating azo reduction, 113.5 and 40.7μmol/min/mg cell dry weight. While no activity was detected when resuspended cells were assayed in the absence of electron donors and glucose.

Azo reduction activity for *B. thetaiotaomicron* was not as great as for *E. faecalis* (Table 3.1). The rate of reduction of amaranth by a washed cell suspension of *B. thetaiotaomicron* was non-quantifiable in the absence of reduced flavins. The maximum rate of reduction was achieved in the presence of FMN (7.2μmol/min/mg cell dry weight). FAD and RF also stimulated azo reduction by *B. thetaiotaomicron*, 4.6 and 5.5μmol/min/mg cell dry weight respectively for amaranth. The rate of reduction of sunset yellow by *B. thetaiotaomicron,* was 7.5μmol/min/mg cell dry weight in the presence of FMN. However when glucose (0.4%) was added to the test system, azoreductase activity decreased to 5.4μmol/min/mg cell dry weight.

When cell free extracts of *E. faecalis* were used in the reduction assay, azo reduction proceeded at a much slower rate (<1%) than reduction by intact cells. Rates of decolouration achieved by both cells and cell free extract were determined and are shown in Table 3.2. Brief exposure of cell-free extracts to oxygen resulted in complete loss of azoreductase activity. Activity was not restored even when later steps were performed under strict anaerobic conditions.

TABLE 3.1 Cofactor requirements of azoreductase

Azo dye	Cofactor	Rate of decolouration (μmol/min/mg cell dry weight)	
		E. faecalis	*B. thetaiotaomicron*
Amaranth	-	NQ	NQ
"	G1	40.7 ± 2.4	NQ
"	FMN	NQ	7.2 ± 1.24
"	G1+FMN	113.5 ± 7.1	3.9 ± 0.7
"	G1+FAD	45.6 ± 3.5	4.6 ± 0.2
"	G1+RF	83.6 ± 5.36	5.5 ± 0.4
Sunset Yellow	-	NQ	NQ
"	G1	79.0 ± 1.5	NQ
"	FMN	3.9 ± 0.17	7.5 ± 0.2
"	G1+FMN	132.5 ± 4.11	5.4 ± 0.25
"	G1+FAD	78.5 ± 1.98	4.6 ± 0.48
"	G1+RF	93.1 ± 3.6	6.3 ± 0.25
Carmoisine	-	NQ	NQ
"	G1	134.0 ± 0.5	NQ
"	FMN	16.5 ± 0.3	19.4 ± 0.9
"	G1+FMN	153.7 ± 1.3	15.3 ± 0.45
"	G1+FAD	108.7 ± 2.4	2.3 ± 0.6
"	G1+RF	122.1 ± 1.6	7.6 ± 1.0

Mean ± SD of 4 independent experiments.
FMN, FAD, RF 0.025mM
G1 Glucose 0.4%
NQ Non-quantifiable (rate less than 1 μmole dye reduced/min/mg cell dry weight)

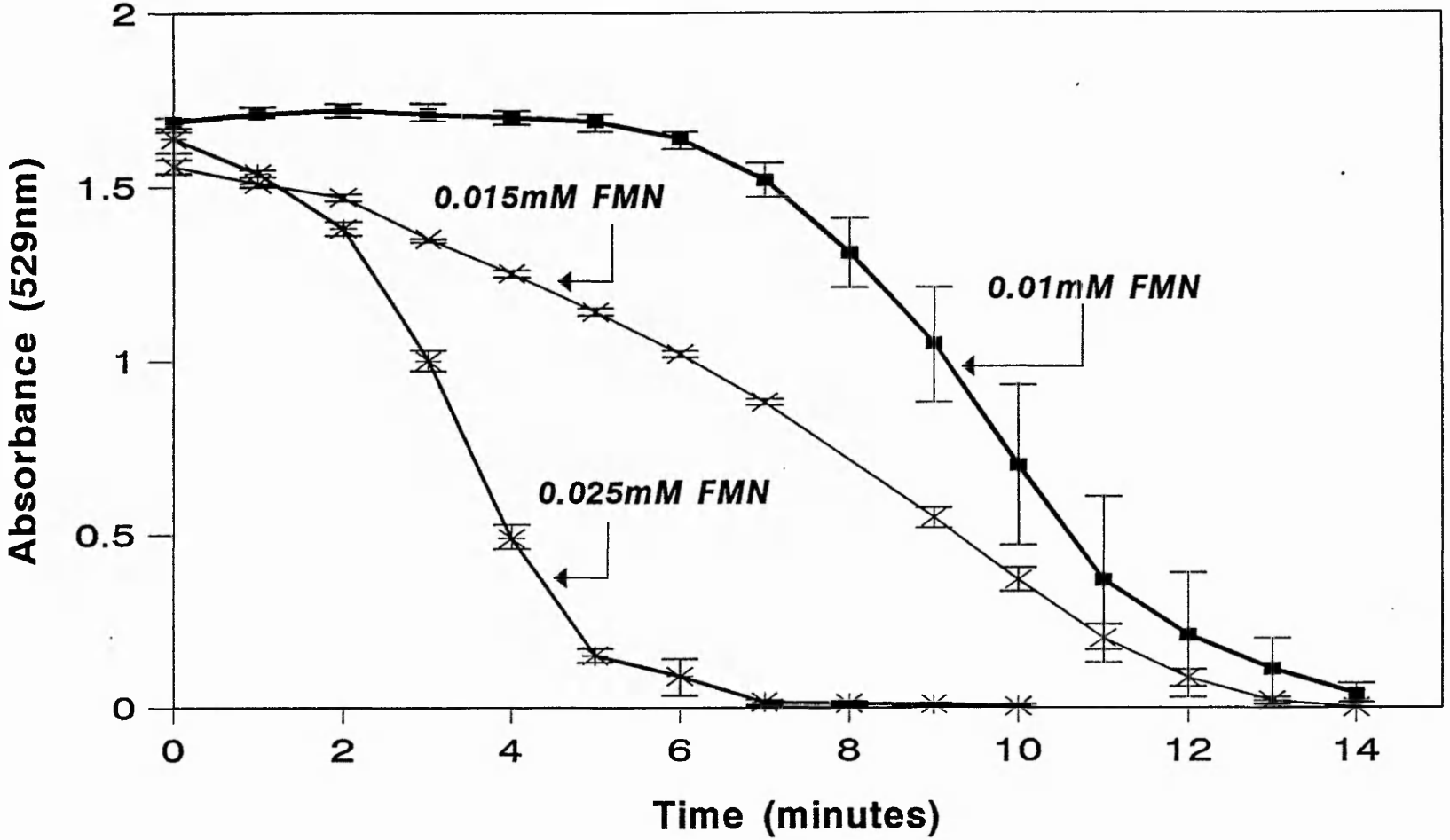


Figure 3.1 Reduction of orange II by *E. faecalis in the presence of increasing concentrations of FMN**

* Washed cell suspension

Data are MEANS +/- SD of 3 independent experiments

TABLE 3.2 Comparison of rates of azo reduction for intact *E. faecalis* cells and cell free extracts

		Rate of decolouration (μmol/min/mg cell dry weight)	
		Intact cell	Cell free extract
Amaranth	Mean	113.5	0.4
	SD	7.1	9.3
Sunset Yellow	Mean	132.5	1.1
	SD	4.1	7.1
Carmoisine	Mean	153.7	2.3
	SD	1.3	5.6

Mean ± SD of 4 independent experiments.

3.2 FACTORS AFFECTING AZO REDUCTION

The effect of various inhibitors on azo dye reduction activity was also studied. There was no marked inhibition by sodium azide, whereas HQNO, a flavoprotein analogue, caused inhibition, resulting in a detectable reduction in azoreductase activity. Rate of reduction of amaranth by *E. faecalis* decreased from 113.5 to 23.9 µmol/min/mg cell dry weight after the addition of HQNO. Similarly, rate of reduction of amaranth by *B. thetaiotaomicron* was decreased from 7.2 to 4.9 µmol/min/mg cell dry weight by the addition of HQNO.

Rate of reduction was shown to vary with culture age (Figure 3.2). Maximum activity was detected while the cells were in the late exponential and early stationary growth stage. Activity decreased as age of culture increased.

Various intestinal micro-organisms were incubated under anaerobic conditions on TSA containing either amaranth or sunset yellow to determine whether they possessed azo reduction activity. Clear zones were observed where the bacteria had reduced the azo dye. The results obtained are shown in Table 3.3. From the results it can be seen that dye reduction capacity of the different micro-organisms varied considerably. These differences may be attributable to variations in growth rates and differences in the production and/or activity of the enzyme.

The degradation of azo dyes by bacteria and cell free extracts was followed spectrophotometrically over time. The spectrometric profile of reduced amaranth by *E. faecalis* is shown in Figure 3.3. An obvious change in UV-visible absorption spectrum of azo dye occurred after treatment with *E. faecalis,* indicating a change in the molecular structure of the azo compound.

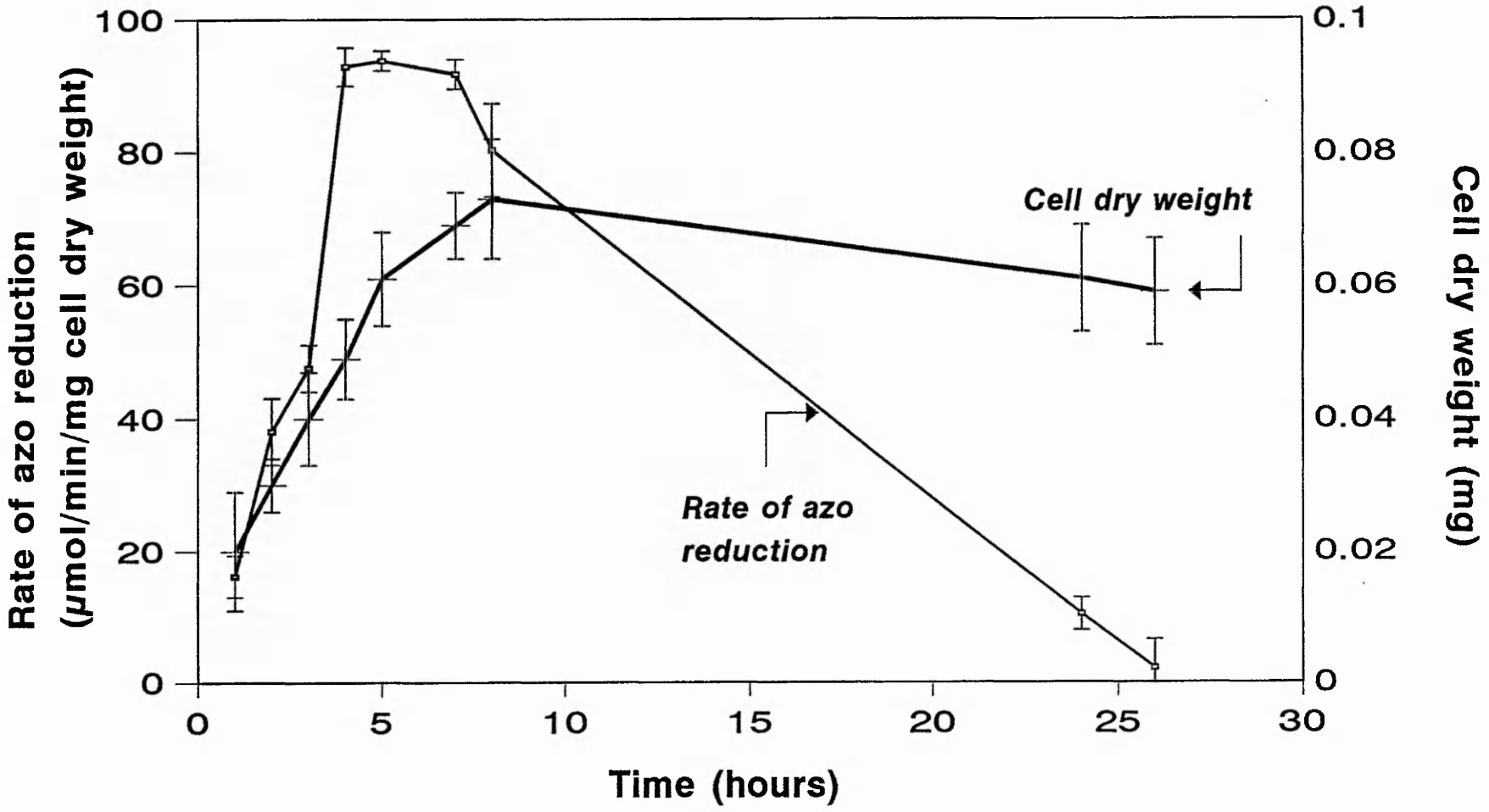


Figure 3.2 Effect of culture age on azoreductase activity of *E. faecalis*

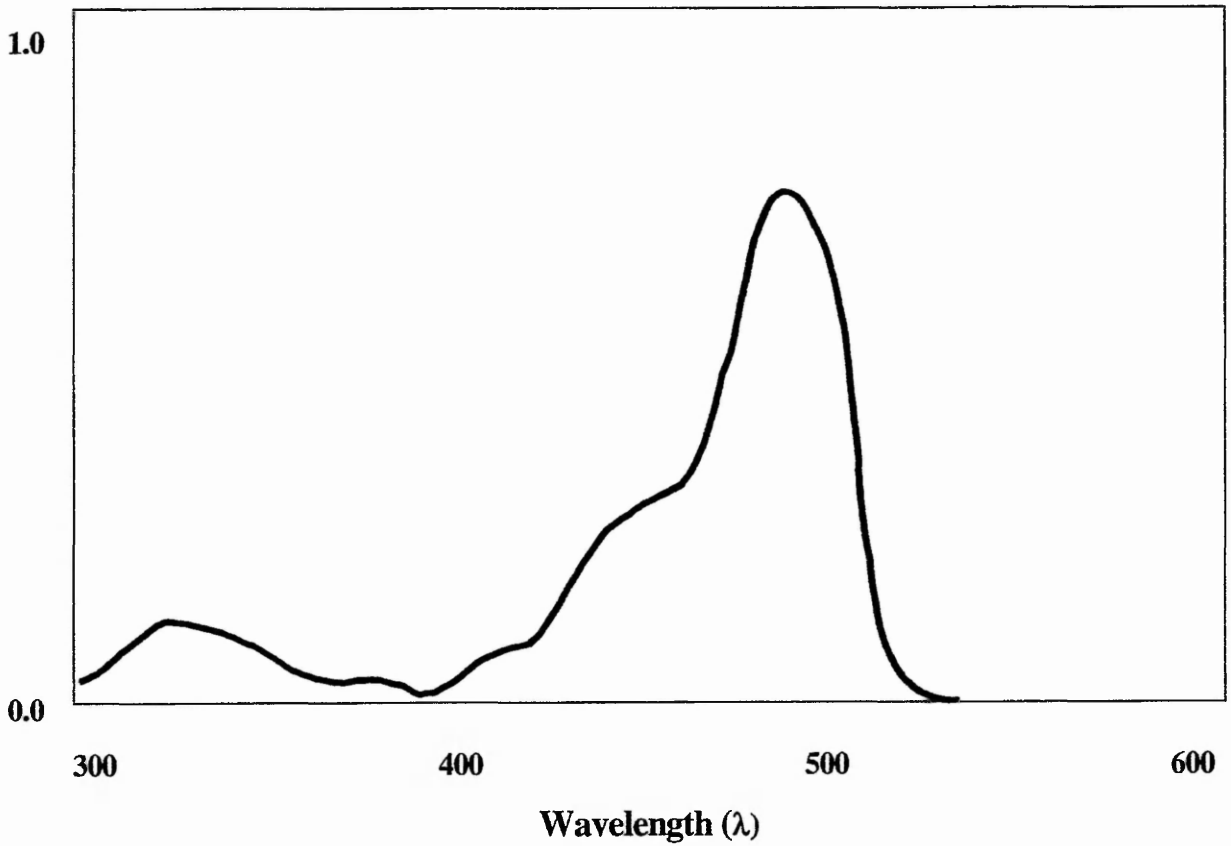
*** Washed cell suspension**

Data are MEANS +/- SD of 3 independent experiments

TABLE 3.3 Azo reduction activity of different microbial groups

Micro-organism	Gram	Aerobic Reduction	Anaerobic Reduction
Enterococcus faecalis	+ve cocci	-	+
Staphylococcus aureus	+ve cocci	-	+
Micrococcus lentus	+ve cocci	-	-
Bacillus subtilis	+ve rod	+	+
Bacillus megaterium	+ve rod	+	+
Neisseria subflava	-ve cocci	-	-
Klebsiella aerogenes	-ve rod	-	-
Escherichia coli	-ve rod	+	+
Enterococcus aerogenes	-ve rod	-	-
Serratia marcescens	-ve rod	-	-
Pseudomonas aeroginosa	-ve rod	-	-
Proteus vulgaris	-ve rod	-	+
Salmonella typhimurium	-ve rod	-	+

Criteria for measuring reduction:
Plates were observed for clearance of the dye amaranth surrounding the bacterial colonies



600nm/min
Source UV/Vis

Figure 3.3. Spectrophotometric profile of *E. faecalis* reduced sunset yellow

3.3 STRUCTURE AND AZO REDUCTION

The influence of structure on azo reduction by *E. faecalis* was investigated (Table 3.4). Azobenzene and its derivative, *p*-phenylazophenol were not reduced. The study revealed that the presence of a sulphonate group on either side of the azo linkage enhanced azo reduction of phenyl/naphthol dyes. Sudan I which has an hydroxy group ortho to the azo linkage on the naphthalene ring but no sulphonate groups had a rate of decolouration of 3.7 μmol/min/mg cell dry weight. Orange II differs from sudan I only by possessing a sulphonate group on the phenyl ring (position 3). The addition of this sulphonate caused the rate of reduction to increase to 18.0 μmol/min/mg cell dry weight. Furthermore the addition of a sulphonate group on the naphthol ring (position 5), as in the case of sunset yellow caused the rate of reduction to increase to 79.0 μmol/min/mg cell dry weight. In contrast the rate of reduction of orange G was non-quantifiable. Orange G differs from the above structures mentioned in that both sulphonate groups are on the naphthol ring (position 5, 7).

A further influence on azo reduction was the position of the sulphonate group on the naphthol ring. Azo dyes with the sulphonate group on position 7 of the naphthol ring were not reduced. For example, ponceau 6R which has a sulphonate group at position 7 of the naphthol ring was not reduced. Amaranth, similar to ponceau 6R except that the sulphonate group is at position 2 rather than 7, had a rate of reduction of 40.7 μmol/min/mg cell dry weight. The rate of reduction of naphthalene/naphthol dyes was not dependent on the presence of a sulphonate group on either side of the azo linkage. Bordeau red, which only has sulphonate groups on the naphthol ring in position 2 and 5, had a similar rate of reduction as amaranth, (41.0 and 40.7 μmol/min/mg cell dry weight respectively). Amaranth has a sulphonate group on both sides of the azo linkage (position 3 on the naphthalene ring, positions 3,6 on the naphthol ring).

TABLE 3.4 Structure activity study of azo dyes

	Rate of decolouration (μmol/min/mg cell dry weight)
MONOAZO DYES	
Derivatives of azobenzene	
Azobenzene	NQ
p-Phenylazobenzene	NQ
Phenyl/Naphthol	
Sudan I	3.79 ± 0.06
Orange II	18.0 ± 0.8
Sunset Yellow	79.0 ± 0.37
Orange G	NQ
Orange I	44.0 ± 1.2
Naphthalene/ Naphthol	
Bordeau Red	41.0 ± 0.7
Amaranth	40.7 ± 0.3
Ponceau 6R	NQ
Carmoisine	95.6 ± 2.5
Eriochrome Blue Black	0.95 ± 0.002
BISAZO DYES	
Sudan III	NQ
Ponceau S	5.24 ± 0.1

Mean ± SD of 4 independent experiments.
NQ Non-quantifiable (rate less than 1 μmole dye reduced/min/mg cell dry weight)
For a guide to dye structures please see inside cover

3.4 PURIFICATION OF AZO DYES

Before toxicity studies were initiated, the azo dyes were examined for the presence of impurities. This was done to assure that the results of the subsequent tests were not compromised by unacceptable levels of impurities within the compounds. While it was not feasible to produce high purity compounds for all the dyes tested, purity for the three main azo dyes studied, namely, amaranth, sunset yellow and carmoisine was enhanced.

3.4.1 Thin Layer Chromatography

A 10µl volume of dye (2µg/µl) in ethanol was spotted onto silica gel plates. Analysis by TLC revealed the presence of many impurities in the commercially produced azo dyes. Using the basic solvent system, methanol:ammonia:water (15:5:80) tailing was observed. The addition of a small amount of pyridine appeared to reduce tailing, while the presence of ammonia in the solvent system appeared to enhance tailing. The presence of higher concentrations of methanol in the system led to slightly increased R_f values for amaranth, carmoisine and sunset yellow. However, this still did not prevent tailing of the spots. Butanol:ethanol:water (50:25:25) was found to be the most efficient system, giving the least tailing and the most distinct spots (Table 3.5).

As a rapid and simple method for determining purity, this system was acceptable. However, the slight tailing effects and incomplete separation of the spots did not make it a confident system for the purification of azo dyes.

3.4.2 Column Chromatography using Chitosan

Both TLC and spectrophotometrical analysis of the products were used for detection of the dye and its impurities after column chromatography using chitosan. Although excellent at adsorbing all the dyes tested, chitosan also adsorbed the impurities in the dyes. None of the solvents tested were suitable for separating the dye from all the impurities present, since the impurities desorbed with the dye. This was probably due to the similarity in structure of the dye and its impurities.

TABLE 3.5 Purification by thin layer chromatography

Dye	Rf Value *	
Amaranth	0.0446	Minor spot
	0.675	Intense spot
Carmoisine	0.613	Minor spot
	0.745	Equal intensity
	0.839	Equal intensity
Sunset Yellow	0.662	Major spot
	0.847	Minor spot

* The Rf ratio correlates data obtained from separations of the same material carried out using different solvent systems.

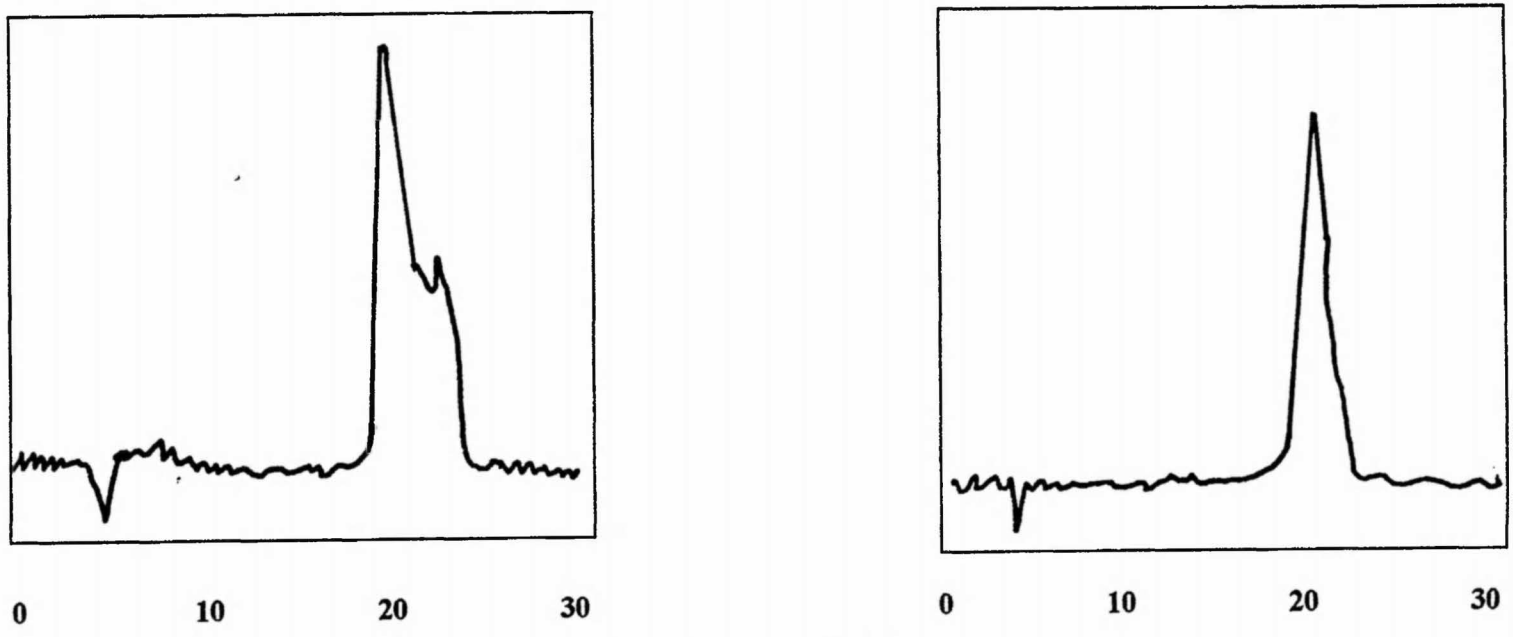
$$Rf = \frac{\text{Distance moved by substance from origin}}{\text{Distance moved by solvent front from origin}}$$

3.4.3 Flash Chromatography

Flash chromatography was successfully used to purify the azo dyes, amaranth, sunset yellow and carmoisine. Butanol:ethanol:water (50:25:25) proved to be a good eluent for the dyes. The pure dyes were isolated from fractions collected from the column, followed by concentration of the extracts with a rotary evaporator. The purity of the dyes was confirmed using TLC, absorption spectrophotometry and HPLC. The cellulose was not reused in case of possible contamination. Although a useful method for purifying the azo dyes, it was not effective in purifying high concentrations of dye. There was only a 10% yield of purified dye with this system.

3.4.4 High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) was used as a sensitive method for detecting impurities in the samples. Purity of the dyes was tested on HPLC by comparing the elution pattern of the dye before and after each purification method. Absence of impurities was confirmed by a single UV-detectable component. Results showed that flash chromatography was the most efficient method for purifying the azo dyes, amaranth, sunset yellow and carmoisine. Figure 3.4 shows typical HPLC traces for amaranth before flash chromatography and after purification. After flash chromatography, purity of amaranth, sunset yellow and carmoisine was determined to be 97.4, 95.2, 90.8 %.



i) before purification by flash chromatography

ii) after purification by flash chromatography

Figure 3.4 HPLC chromatogram of amaranth

3.5 DIFFERENTIAL KILLING ASSAY

3.5.1 Activity of azo dyes in the Differential Killing Assay

Differential killing assays provide a simple means of detecting chemically induced DNA damage. The test used in this study was initially based on two strains of *E. coli*: WP2, a repair proficient strain and CM871 *uvr*A *rec*A *lex*A. As the *lex*A mutation suppresses the spontaneous inviability of *rec*A strains of *E. coli* without affecting their repair deficiency, CM871 did not suffer from poor growth. At a later stage a further two strains of *E. coli* were used in the differential killing assay. *E. coli* AB1157, a repair proficient strain and *E. coli* BH20, *fpg-1*::Kn^r. For the differential killing assay a result was considered positive when the DNA repair-deficient strain was preferentially inhibited or killed to a significant extent as compared with the repair-proficient strain. If survival of *E. coli* CM871 was consistently four-fold less than survival of *E. coli* WP2, results were considered positive. Results were expressed as coefficients of survival (CS)

$$= \frac{\%\text{ survivors of repair deficient strain}}{\%\text{ survivors of repair proficient strain}}$$

Within the limits of the assay, survival coefficients below 0.3 were taken as positive, values between 0.3 and 0.85 were weakly positive and values > 0.85 as negative. Compounds were deemed cytotoxic where there was excessive kill (2-fold reduction in cell numbers) of the wild type *E. coli* strain. Unless otherwise stated, results are given for the *E. coli* WP2 & CM871 pair.

After incubation with *E. faecalis*, the cleavage products of sunset yellow (5mM) and amaranth (5mM) were found to be genotoxic (Figure 3.5a). Carmoisine was marginally genotoxic after azo reduction, but survival of *E. coli* CM871 was not consistently four-fold less than survival of *E. coli* WP2. Concentrations of azo dye above 10mM were required to produce a genotoxic effect after reduction by *B. thetaiotaomicron* (Figure 3.5b). Unreduced dyes were not genotoxic in this assay. The dyes were not genotoxic using tester pairs *E. coli* AB1157 & BH20.

When the differential kill assay was carried out under strictly anaerobic conditions, none of the reduced dyes exhibited genotoxicity. At the maximum study concentration

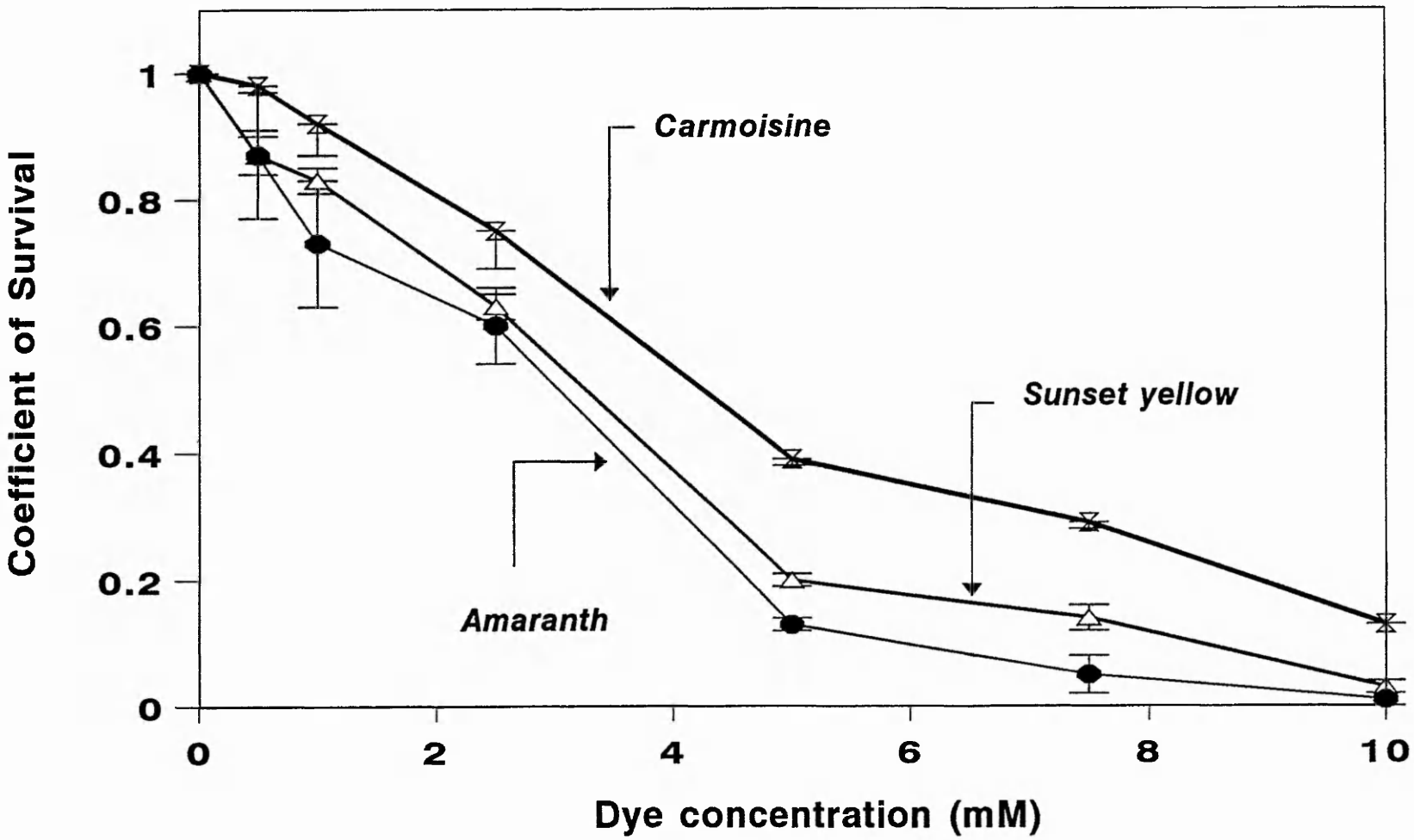


Figure 3.5a Genotoxicity exhibited by azo dyes after reduction by *E. faecalis

* Washed cell suspension

Data are means +/- SD of 4 independent experiments

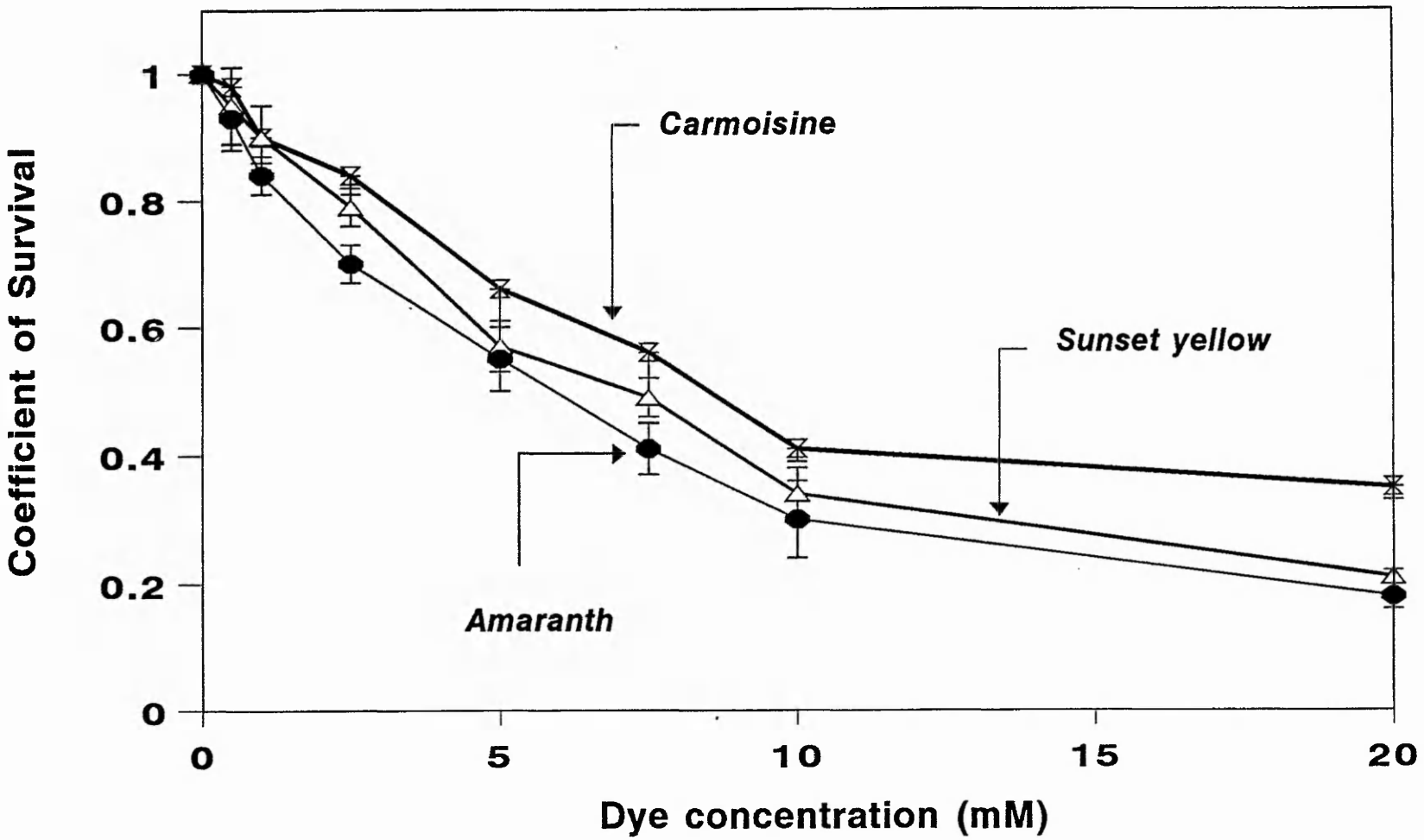


Figure 3.5b Genotoxicity exhibited by azo dyes after reduction by *B. thetaiotaomicron

*** Washed cell suspension**

Data are means +/- SD of 4 independent experiments

(100mM), the coefficient of survival for reduced amaranth, sunset yellow and carmoisine was 0.86±0.05, 0.87±0.02 and 0.89±0.2 respectively.

Dyes were tested pre- and post- purification by flash chromatography and thin layer chromatography. After reduction by *E. faecalis*, pre- and post- purified amaranth (5mM) had a CS of 0.13±0.01 and 0.15±0.02 respectively. Similarly sunset yellow (5mM) had a CS of 0.21±0.01 and 0.22±0.03 respectively. The CS for pre- and post- purified reduced carmoisine was 0.39±0.01 and 0.37±0.02. Thus as the purified material showed similar genotoxicity to the impure dye, genotoxicity must be due to the reduced dye products and not to any of their impurities.

Twelve further azo dyes were studied for their ability to cause genotoxicity in the bacterial assay system. The dyes selected were both the monoazo type and bisazo type based on amaranth, sunset yellow and carmoisine. Both azobenzene and its derivative, p-phenylazophenol were non-genotoxic at the concentrations studied (0.5mM - 50mM). Subsequent incubation with *E. faecalis* for 1 hour (to mimic azo dye reduction) had no effect on their genotoxicity.

Of the monoazo phenyl-naphthol dyes tested only orange I exhibited any genotoxicity (Figure 3.6a). This dye differs from the other phenyl-naphthol dyes studied in that the hydroxy moiety is para to the azo linkage. After incubation with *E. faecalis* all phenyl-naphthol dyes studied exhibited genotoxicity (Figure 3.6b). Genotoxicity of orange I increased after azo reduction by *E. faecalis*. The structures of the dyes sudan I and orange II differ only in the presence of a sulphonate group on the phenyl ring of orange II. They showed similar dose responses, having CS of 0.69±0.04 and 0.67±0.06 at 0.5mM for sudan I and orange II respectively. Orange G which was not reduced by *E. faecalis* was non-genotoxic. These results indicated that reduction of the azo dye to the naphthol ring played a major role in the genotoxicity of these dyes.

Eriochrome blue black was the only naphthol-naphthol dye studied. It was genotoxic in its unreduced form (Figure 3.6a). It also retained its genotoxicity after azo reduction by *E. faecalis* (Figure 3.6c). This may be due to the release of two hydroxy naphthol

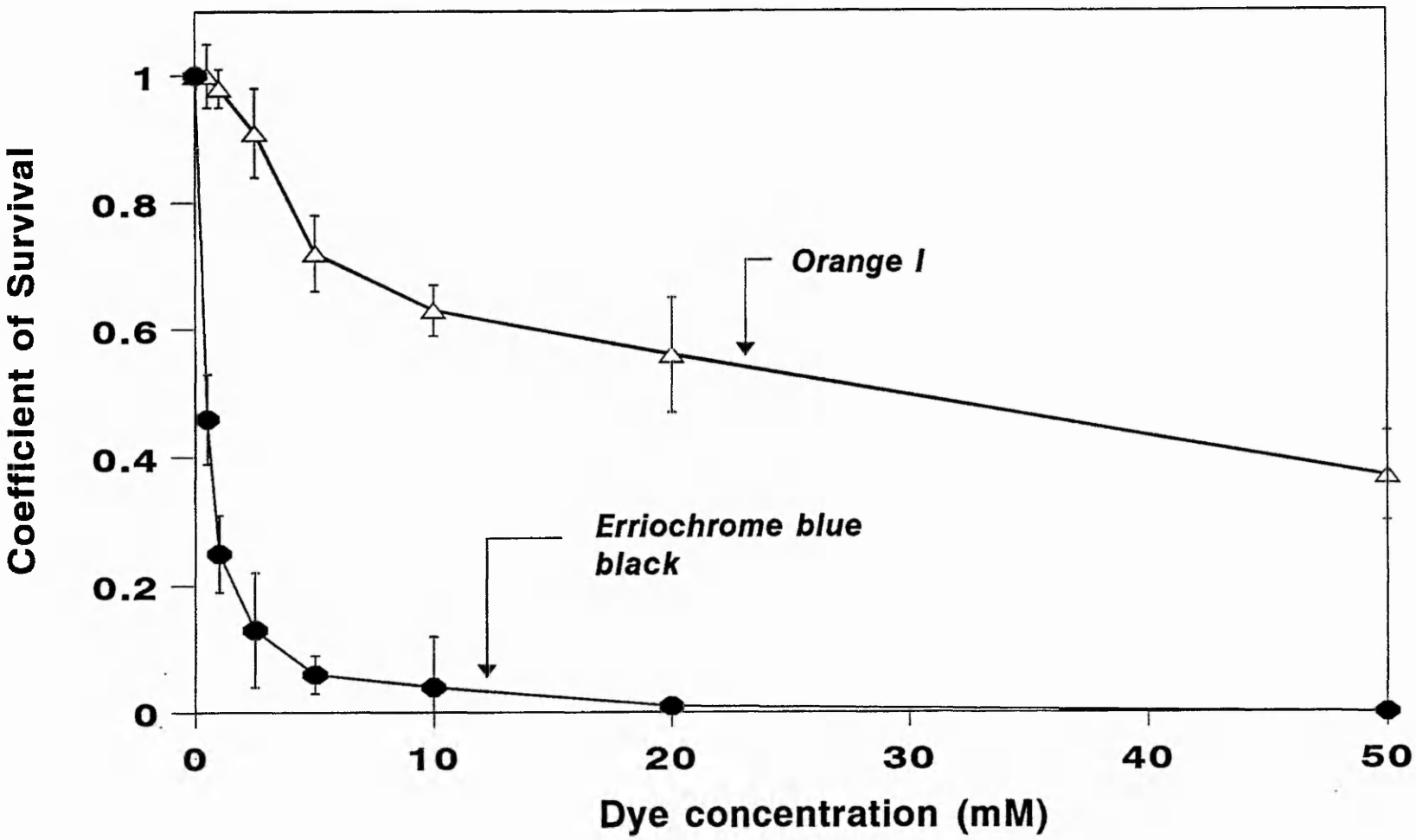


Figure 3.6a Genotoxicity exhibited by structurally related azo dyes

Data are means +/- SD of 4 independent experiments

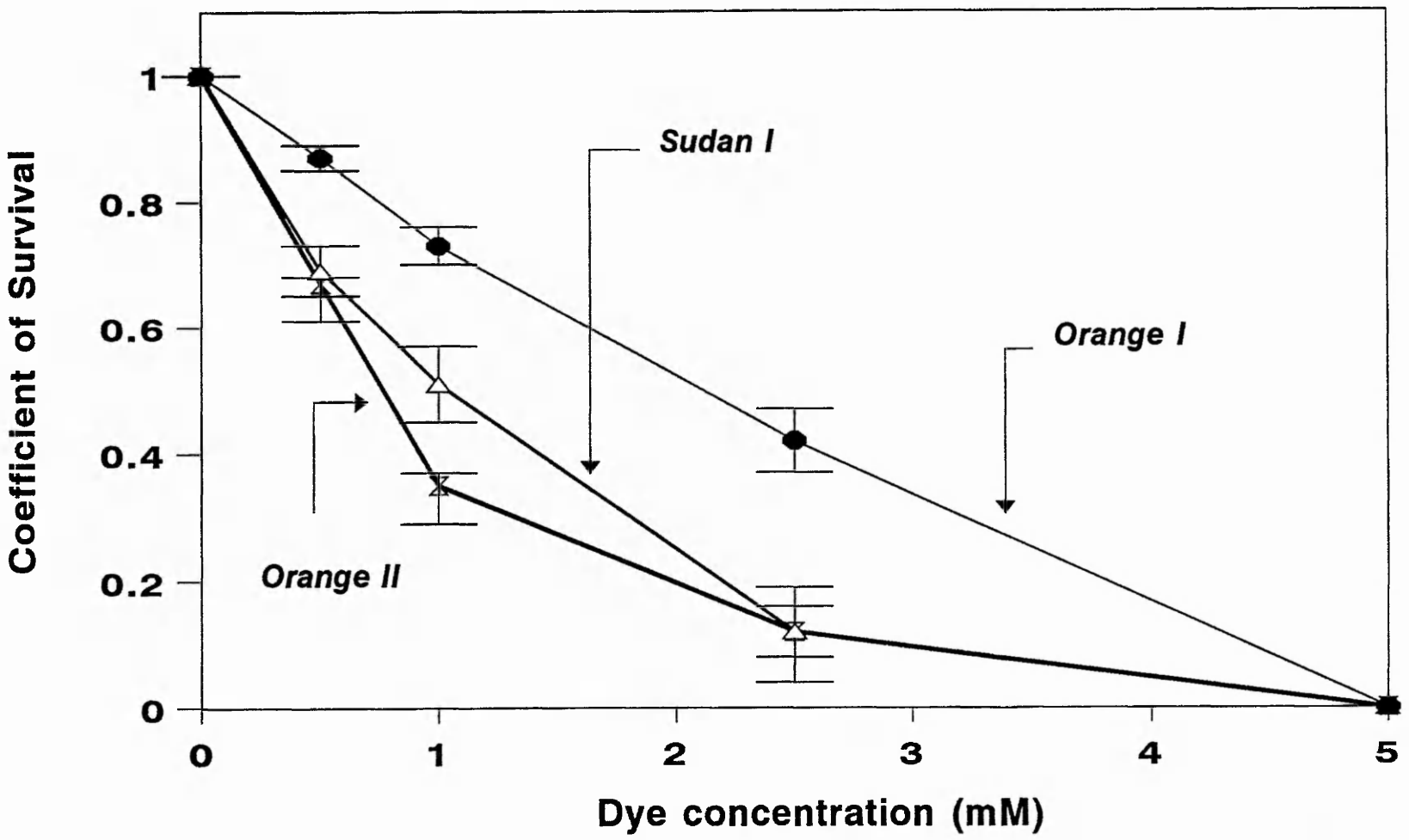


Figure 3.6b Genotoxicity exhibited by phenyl/naphthol azo dyes after reduction by *E. faecalis

* Washed cell suspension

Data are means +/- SD of 4 independent experiments

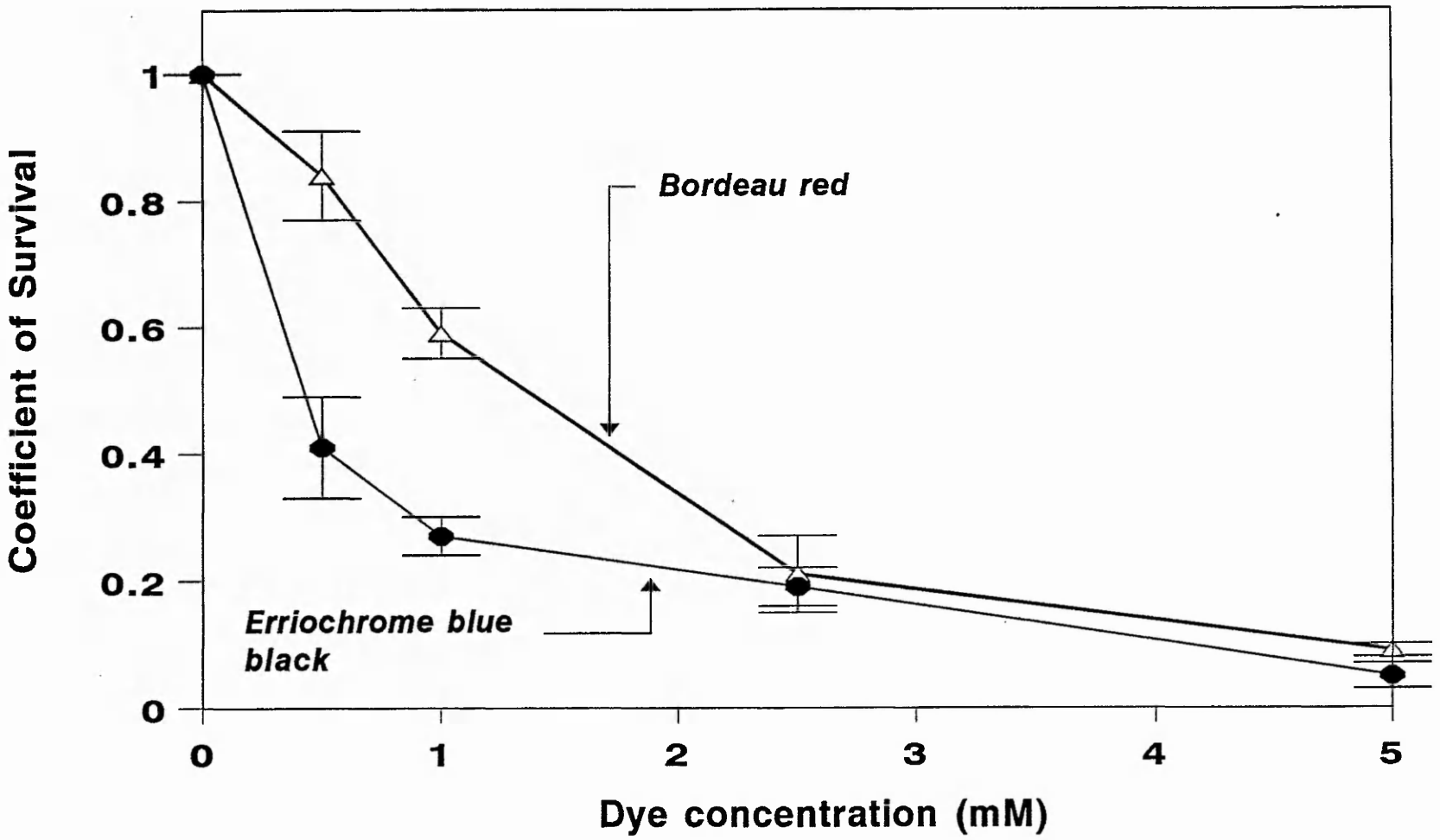


Figure 3.6c Genotoxicity exhibited by naphthalene/naphthol azo dyes after reduction by *E. faecalis

* Washed cell suspension

Data are means +/- SD of 4 independent experiments

moieties. The naphthalene-naphthol dyes bordeau red and amaranth had similar genotoxic profiles, both requiring azo reduction to become genotoxic (Figure 3.6c). This compares favourably with the similarity in their structure and azo reduction rates by *E. faecalis*. Ponceau 6R, which is not reduced by *E. faecalis*, was non-genotoxic at the concentrations studied (0mM - 50mM), both pre- and post- incubation with *E. faecalis*. These results suggest that the breakage of the azo linkage, visualised by decolouration of the dye, is an important step in activating phenyl- or naphthalene-naphthol azo dyes.

3.5.2 Effect of incubation time on azo dyes reduced with *E. faecalis*

The genotoxicity of azo dyes increased with continued incubation with *E. faecalis* over an initial 3 hour incubation (Table 3.6). The increase in genotoxicity may be due to reduction of the azo bond by *E. faecalis* and the subsequent production of aromatic amines. The instability of these end-products was demonstrated by the gradual decrease in genotoxicity with time. After 1 hour incubation with *E. faecalis* amaranth (5mM) had a CS of 0.08±0.09, whereas the CS had increased to 0.41±0.03 after 24 hour incubation with *E. faecalis* (Table 3.6). This trend was also shown with sunset yellow and carmoisine. As indicated previously, reduced amaranth appeared more genotoxic than reduced sunset yellow. After 24 hours incubation with *E. faecalis*, sunset yellow (5mM) and carmoisine (5mM) had CS of 0.93±0.05 and 0.97±0.04 respectively. Instability may be due to chemical oxidation, photo-oxidation or further biological deactivation by *E. faecalis*. This instability was visualised by the gradual appearance of an orange-brown pigment for both reduced amaranth and sunset yellow and corresponded with a decrease in genotoxicity.

The stability of filtered reduced dyes was shown to decrease with time (Table 3.6). All reduction products developed a brown colouration several hours after reduction. This colouration corresponded with a gradual decrease in genotoxicity. After 24 hours the reduced supernatants of amaranth, sunset yellow and carmoisine had CS of 0.65±0.06, 0.95±0.01 and 1.00±0.09 respectively. This clearly indicates the relative instability of the filtered reduced dyes.

TABLE 3.6 Effect of incubation time on azo dyes reduced with *E. faecalis*

Time prior to genotoxicity testing (h)	Coefficient of Survival*		
	Amaranth (5mM)	Sunset yellow (5mM)	Carmoisine (5mM)
Prolonged incubation with *E. faecalis*			
0	0.15±0.01	0.19±0.01	0.35±0.02
0.5	0.12±0.05	0.16±0.09	0.34±0.08
1.0	0.08±0.09	0.15±0.01	0.29±0.08
1.5	0.04±0.07	0.17±0.02	0.25±0.01
2.0	0.01±0.01	0.18±0.04	0.23±0.02
3.0	0.08±0.06	0.34±0.07	0.28±0.0
4.0	0.08±0.04	0.40±0.08	0.33±0.05
5.0	0.34±0.00	0.60±0.04	0.45±0.07
24.0	0.41±0.03	0.93±0.05	0.97±0.04
Stored at 37^0 C after filtration of *E. faecalis*			
0	0.12±0.07	0.20±0.03	0.35±0.02
0.5	0.08±0.01	0.20±0.01	0.28±0.01
1.0	0.12±0.01	0.22±0.02	0.34±0.08
1.5	0.09±0.02	0.25±0.08	0.39±0.6
2.0	0.025±0.08	0.290.02	0.48±0.07
3.0	0.09±0.02	0.42±0.02	0.52±0.01
4.0	0.19±0.05	0.47±0.03	0.57±0.06
5.0	0.48±0.03	0.52±0.01	0.66±0.06
24.0	0.65±0.06	0.95±0.01	1.00±0.09

Mean ± SD of 4 independent experiments

* CS = $\frac{\%\text{ survivors of repair deficient strain}}{\%\text{ survivors of repair proficient strain}}$

3.5.3 Genotoxicity of azo dyes after chemical reduction

Azo dyes were reduced by chemical means to determine whether chemical reduction resulted in end-products which were more or less genotoxic than those produced after bacterial reduction. In this study sodium dithionite was added at 0.05mg/ml. Higher concentrations of dithionite were too toxic and resulted in cell death of *E. coli* WP2, the repair proficient strain. Sunset yellow (5mM) and amaranth (5mM) were genotoxic in the differential assay using *E. coli* tester pair WP2 & CM871, after preincubation and reduction by dithionite (Figure 3.7). As in the case of bacterial reduction, reduced amaranth was more genotoxic than sunset yellow, with CS of 0.09 ±0.08 and 0.15±0.01 respectively. Genotoxicity of dithionite-reduced carmoisine (CS, 0.23±0.03) compared favourably with results obtained after bacterial reduction (CS, 0.35±0.08). The concentration of dye was standardised at 5mM. In comparison studies it was important to use the same concentration for all dyes and 5mM was the concentration which gave coefficient of survivals within the limits specified.

While dithionite is a mild reducing agent, it remains to be demonstrated that it does not cause chemical changes other than the type of azo reduction that would occur in the intestine *in vivo*. Reduction by stannous chloride (0.25g/ml) did not increase genotoxicity of the dyes as compared to bacterial reduction.

3.5.4 Genotoxicity after microsomal activation

Of the azo dyes tested only eriochrome blue black exhibited genotoxicity in the presence of rat S9 without prior bacterial or chemical reduction. The addition of S9 to the preincubation mix depressed the genotoxic activity of the reduced dyes. *E. faecalis*-reduced amaranth (5mM) had a CS of 0.13±0.03, the addition of S9 to the preincubation mix resulted in deactivation of the reduced dye, CS of 0.32±0.01. Similarly, *E. faecalis*-reduced sunset yellow (5mM) was deactivated by the presence of S9 in the preincubation mix. The CS were 0.2±0.02 and 0.31±0.03 for reduced dye and reduced dye plus S9 respectively. Carmoisine was deactivated also by the presence of S9, CS was 0.36±0.03 and 1.00±0.07 for reduced dye and reduced dye plus S9. It is possible that the reduced

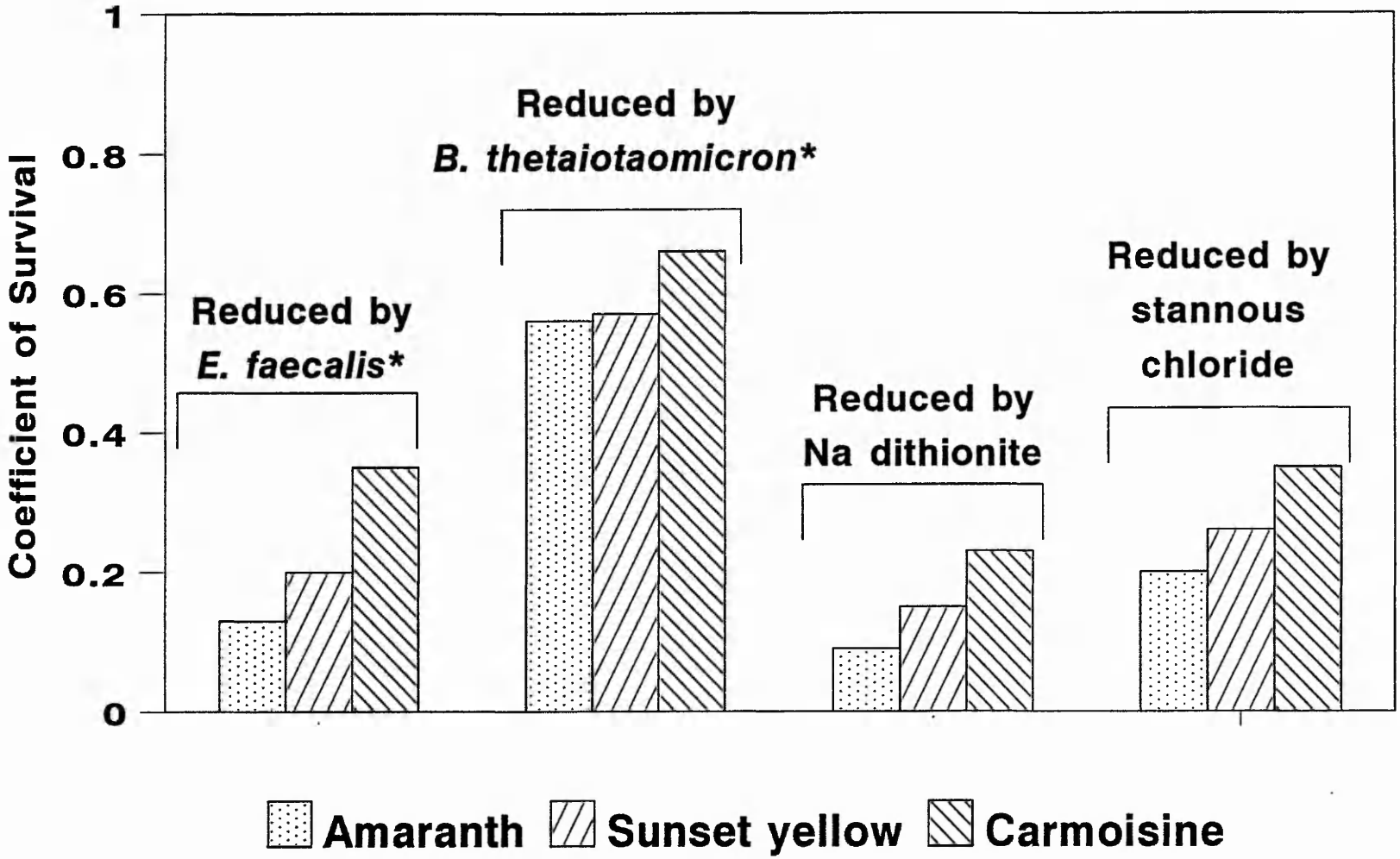


Figure 3.7 Genotoxicity of azo dyes after azo-fission

* Washed cell suspension

Data are means +/- SD of 4 independent experiments

dye metabolites were inactivated by reaction with proteins and other substances in S9 and/or by further metabolic inactivation such as further reduction.

Similar studies were carried out using *E. coli* AB1157 (repair proficient) and BH20 (repair deficient). As shown previously none of the reduced dyes were genotoxic using these strains. The presence of S9 in the reaction mix had no effect on the activity of the reduced dyes.

3.5.5 Predicted end-products of azo reduction

The instability of many azo-fission products has prevented them from being isolated and identified. Table 3.7 lists the possible azo reduction end-products which were either available commercially or were kindly donated by Dr. I. Coutts, Department of Chemistry, The Nottingham Trent University.

Table 3.8 shows the compounds and the maximum concentrations tested that were non genotoxic in the tester strains. 1-Amino-2-naphthol, the predicted end-product from reduction of sudan I, orange II, EBB and sudan III was particularly genotoxic (Figure 3.8a). It had to be tested at relatively low levels (0.05mM - 0.1mM) compared to the other end-products studied. This correlated well with data obtained from previous studies on *E. faecalis*-reduced dyes, indicating that 1-amino-2-naphthol is the genotoxic agent in sudan I, orange II, EBB and sudan III. 1-Amino-2-naphthol-4-sulphonate, predicted as being the second azo reduction product of EBB, was also tested for genotoxicity. It also exhibited a positive response in the differential kill assay.

The two possible end-products for azo reduction of amaranth (1-amino-2-naphthol-3,6-disulphonate and 1-amino-naphthalene-4-sulphonate) were both available for study. 1-Amino-naphthalene-4-sulphonate did not elicit a response in any *E. coli* strains. In contrast 1-amino-2-naphthol-3,6-disulphonate produced a positive dose response (Figure 3.8b). The presence of the two sulphonate groups appeared to diminish genotoxicity as compared with 1-amino-2-naphthol. Similarly, 1-amino-2-naphthol-6-sulphonate exhibited positive genotoxicity at concentrations >4mM. This compares favourably with

the genotoxicity of *E. faecalis*-reduced sunset yellow. Sulphanilic acid, the other predicted end-product of sunset yellow produced no response in the assay (Figure 3.8c). When these compounds were tested in the differential kill assay using *E. coli* AB1157 & BH20 pairs no genotoxicity was observed.

TABLE 3.7 Predicted end-products from azo reduction

Azo dye	Predicted azo reduction product	
Sudan I	1-amino-2-naphthol*	amino-benzene
Orange II	1-amino-2-naphthol*	sulphanilic acid*
Eriochrome blue black	1-amino-2-naphthol*	1-amino-2-naphthol-4-sulphonate*
Sudan III	1-amino-2-naphthol*	amino-azo-benzene*
Amaranth	1-amino-2-naphthol-3,6-disulphonate#	1-amino-naphthalene-4-sulphonate*
Sunset yellow	1-amino-2-naphthol-6-sulphonate#	sulphanilic acid*
Carmoisine	1-amino-2-naphthol-7-sulphonate	1-amino-naphthalene-4-sulphonate*

* Compounds are commercially available

Compounds were kindly synthesised by Dr. I. Coutts

TABLE 3.8 Maximum non-toxic concentrations tested that did not express genotoxicity in *E. coli* WP2 & CM871

Predicted azo reduction product	Maximum non-toxic concentration (mM)
1-Amino-2-naphthol*	0.05
1-Amino-2-naphthol-4-sulphonate*	5.0
1-Amino-2-naphthol-6-sulphonate#	2.0
1-Amino-2-naphthol-3,6-disulphonate#	0.5
Sulphanilic acid*	50.0
1-Amino-naphthalene-4-sulphonate*	50.0

* Compounds are commercially available

Compounds were kindly donated by Dr. I. Coutts

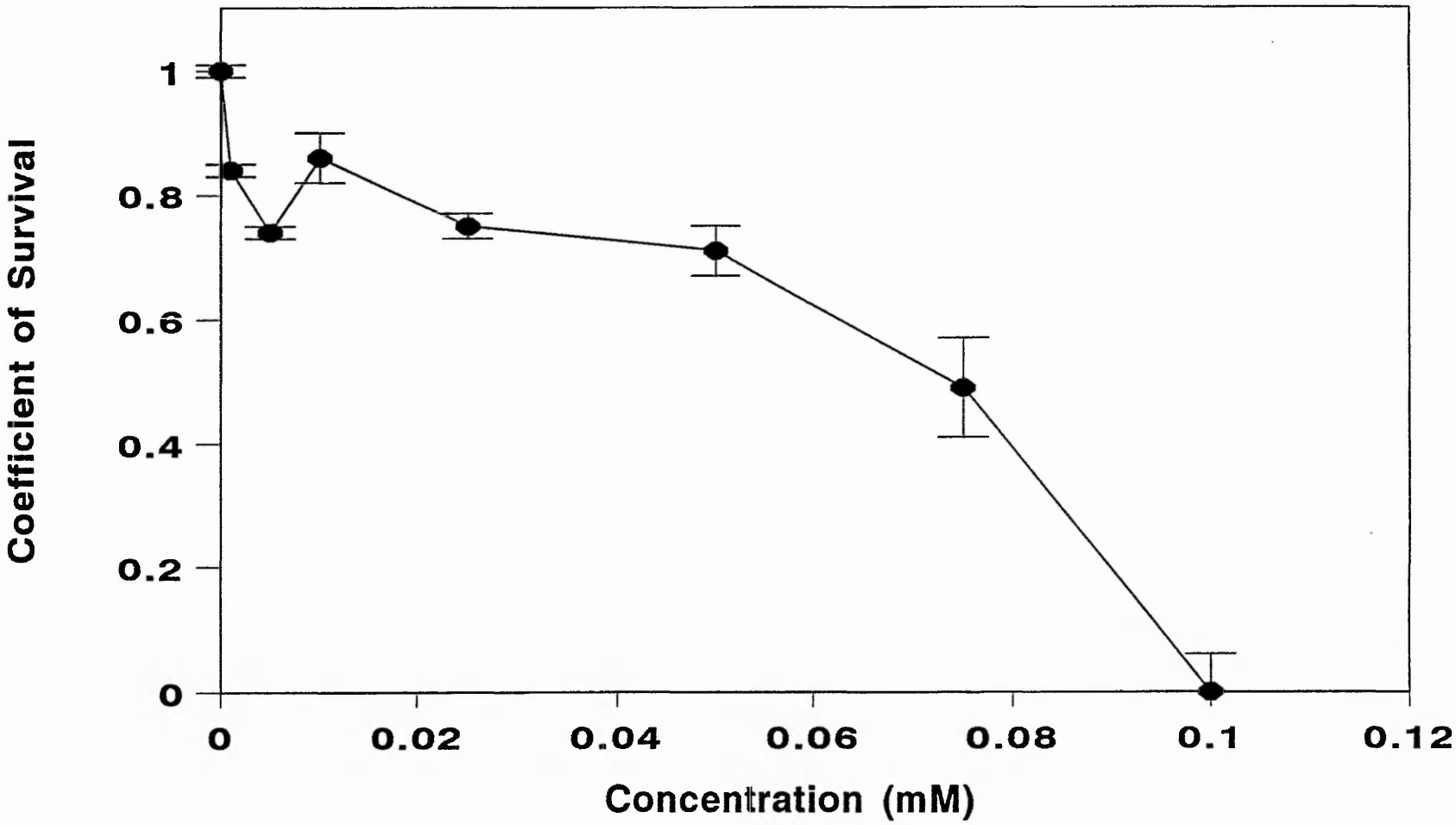


Figure 3.8a Genotoxicity exhibited by 1-amino-2-naphthol

Data are means +/- SD of 4 independent experiments

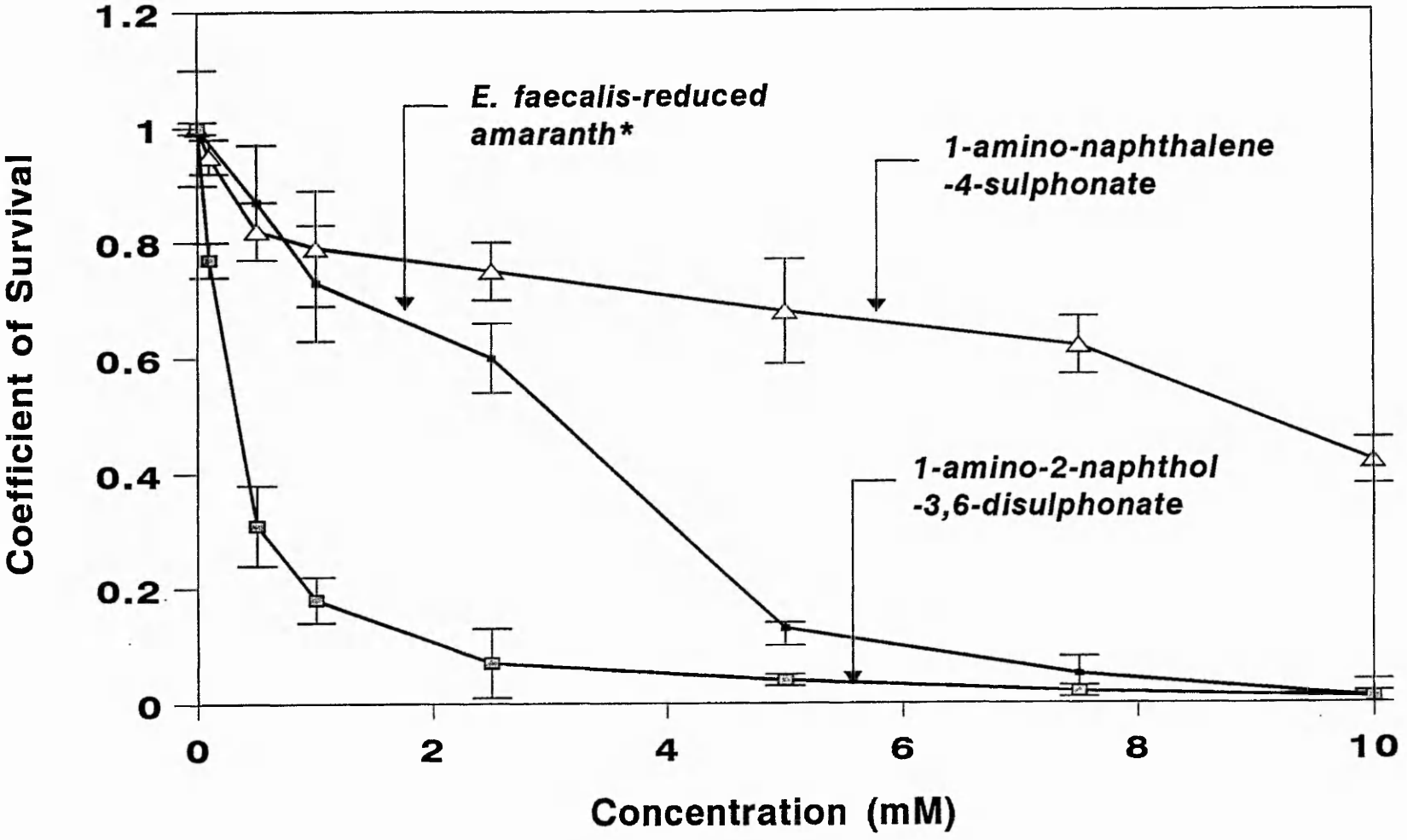


Figure 3.8b Genotoxicity exhibited by reduced amaranth and its predicted end-products

*** Washed cell suspension**
Data are means +/- SD of 4 independent experiments

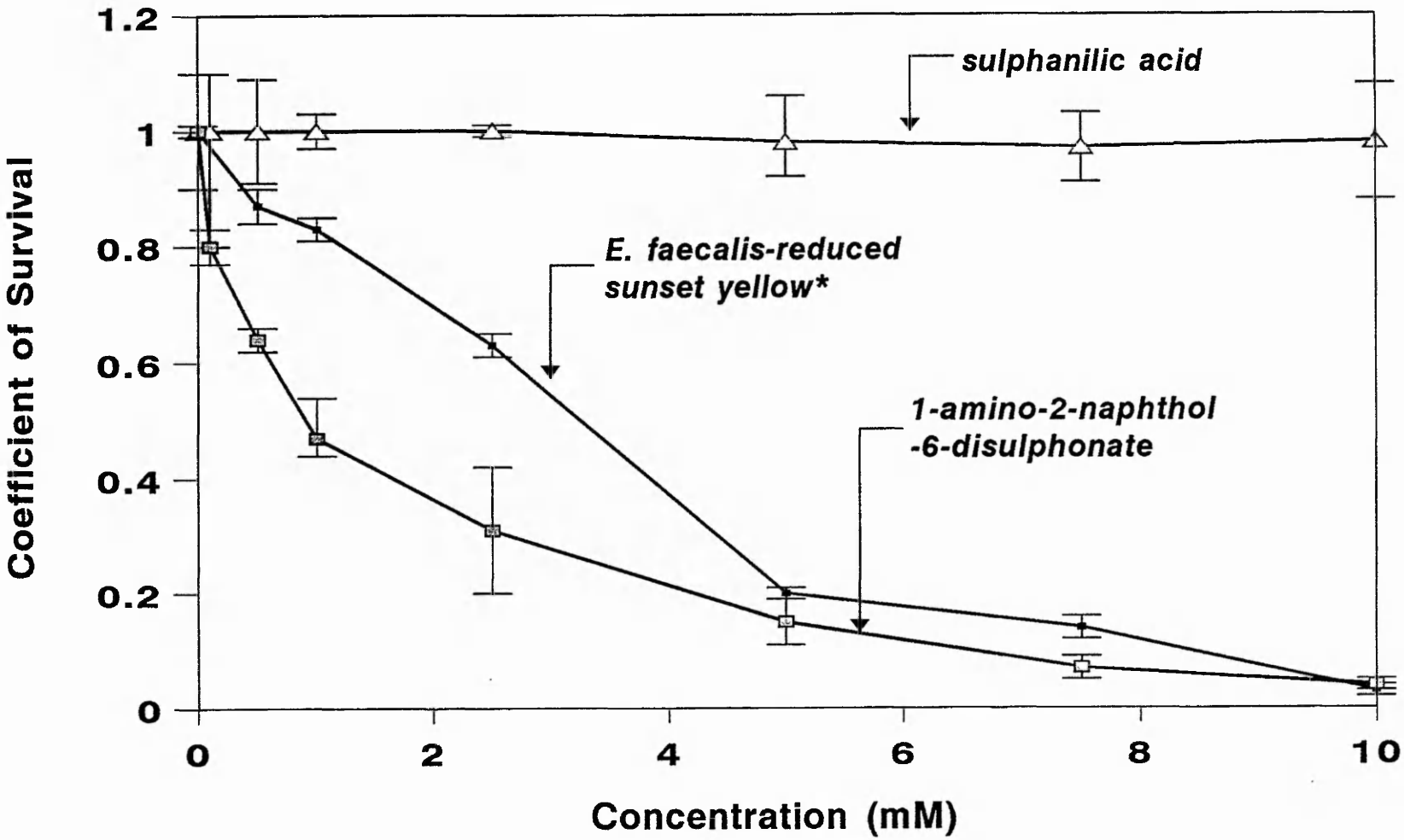


Figure 3.8c Genotoxicity exhibited by reduced sunset yellow and its predicted end-products

*** Washed cell suspension**
Data are means +/- SD or 4 independent experiments

3.5.6 Effect of incubation on azo dye metabolites

Genotoxicity of possible azo fission products was shown to be time dependent (Table 3.9). The concentration used was dependent on the genotoxicity of the test compound and was based on the concentration which gave coefficient of survivals within the limits specified.

1-Amino-2-naphthol became more genotoxic after incubation with either *E. faecalis* or *B. thetaiotaomicron.* Prolonged incubation of 1-amino-2-naphthol (0.025mM) with *E.* faecalis resulted in genotoxicity increasing from CS of 0.48±0.02 at 1 hour to 0.02±0.01 at 4 hours. After 24 hours incubation with *E. faecalis,* the test agent was toxic to the repair proficient strain *E. coli* WP2. Similarly, after prolonged incubation with *B. thetaiotaomicron,* 1-amino-2-naphthol caused excessive kill of the wild type strain in the differential kill assay.

1-Amino-2-naphthalene-4-sulphonate, which was non-genotoxic at the concentrations studied (0 - 50mM), remained inactive after prolonged incubation with either *E. faecalis* or *B. thetaiotaomicron.*

TABLE 3.9 Stability of predicted azo fission products after prolonged bacterial incubation

Time prior to genotoxicity testing (h)	Coefficient of Survival*		Coefficient of Survival*	
	E. faecalis incubation		*B. thetaiotaomicron* incubation	
	1-Amino-2-naphthol (0.025mM)	1-Amino-naphthalene-4-sulphonate (50mM)	1-Amino-2-naphthol (0.025mM)	1-Amino-naphthalene-4-sulphonate (50mM)
0	0.7 ± 0.04	0.79 ± 0.06	0.93 ± 0.06	0.95 ± 0.09
0.5	0.49 ± 0.05	0.65 ± 0.01	0.87 ± 0.02	0.68 ± 0.07
1.0	0.48 ± 0.02	0.66 ± 0.08	0.43 ± 0.01	0.68 ± 0.03
1.5	0.34 ± 0.03	0.62 ± 0.04	0.44 ± 0.06	0.74 ± 0.06
2.0	0.18 ± 0.04	0.61 ± 0.07	0.45 ± 0.03	0.67 ± 0.10
3.0	0.07 ± 0.06	0.72 ± 0.04	0.34 ± 0.01	0.91 ± 0.08
4.0	0.02 ± 0.01	0.55 ± 0.07	0.01 ± 0.07	0.89 ± 0.04
5.0	Cytotoxic#	0.57 ± 0.08	0.01 ± 0.03	0.72 ± 0.05
24.0	Cytotoxic#	1.00 ± 0.01	Cytotoxic#	0.87 ± 0.03

Mean ± SD of 4 independent experiments

* CS = $\frac{\text{\% survivors of repair deficient strain}}{\text{\% survivors of repair proficient strain}}$

\# Cytotoxic: Excessive wild type kill

3.5.7 Protection by antioxidants

Dietary intake of natural antioxidants may be an important aspect of the body's defence mechanisms against active oxygen species (Ames, 1983). The 6 dietary compounds studied were β-carotene, retinoic acid, retinol, ascorbic acid, ferulic acid and caffeic acid. Also studied were chlorophyllin (a water soluble derivative of chlorophyll) and l-ergothioneine (which is present in a variety of fungi, including *Neurospora crassa*). These have all been reported to be effective antioxidants and anti-mutagens against various chemical mutagens and dietary mixtures.

Caffeic acid is a phenolic compound widely distributed in plants and it has been shown to produce strand breaks in DNA (Inoue *et al.*, 1992). It had no protective effect against damage by amaranth (5mM) at the concentrations studied (0.1mM - 100mM). In fact caffeic acid, at concentrations greater than 5.0mM was found to exhibit a positive response in the differential kill assay using *E. coli* tester strains WP2 & CM871. Ferulic acid, which is structurally similar to caffeic acid, also elicited a positive response in the differential kill assay at concentrations greater than 5.0mM. It was shown to be non-protective against damage by-reduced amaranth (5mM) at concentrations between 0.1mM to 100mM.

Although retinoids are important anti-mutagens (Hartman and Shankel, 1990), excess retinoids have been shown to be toxic and teratogenic (Kann, 1982). My investigations showed retinoids to be non-genotoxic and non-protective. Concentrations studied ranged from 0.1mM to 100mM. Protective properties were not detected for β-carotene (0.1mM - 100mM) and chlorophyllin (0.1mM - 100mM).

Ascorbic acid, a scavenger of many free radicals was protective against reduced amaranth (5mM) damage at concentrations of 5mM and above (Figure 3.9). Ascorbate alone produced no response in the assay. α-Tocopherol was found to be highly protective against reduced amaranth (5mM) induced-damage, with CS increasing from 0.14±0.03 to 0.38±0.02 at 0 and 0.05 mM added α-tocopherol.

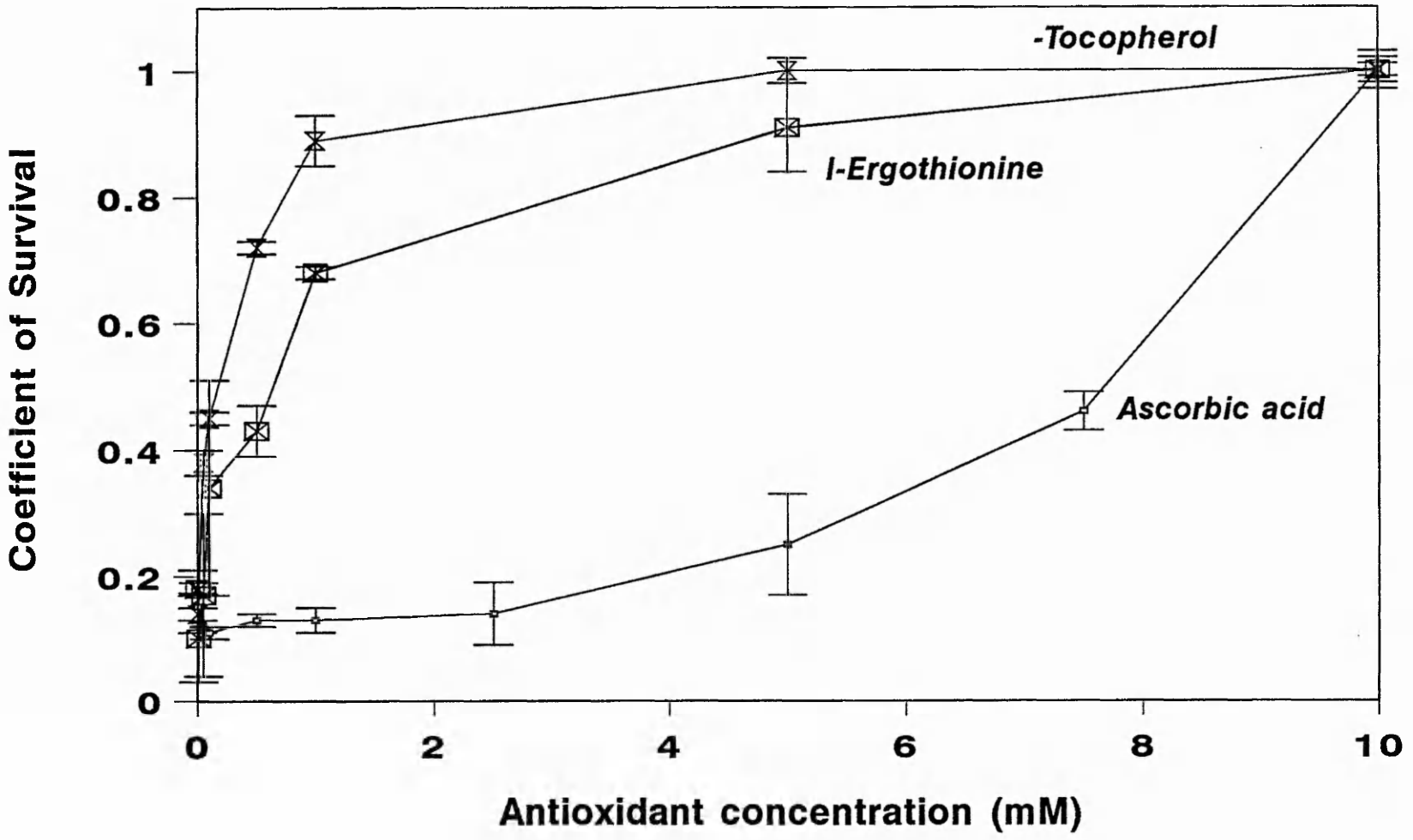


Figure 3.9 Protective effect of antioxidants on *E. faecalis reduced amaranth (5mM)**

* Washed cell suspension

Data are means +/- SD of 4 Independent experiments

L-Ergothioneine significantly inhibited damage by reduced amaranth (5mM) at concentrations of 0.1mM and above (Figure 3.9). L-Ergothioneine is a potentially important defence against electrophiles and free radicals (Hartman and Hartman, 1987). These results suggest that reduced azo dyes produce hydroxy radicals which are eliminated by l-ergothioneine or that l-ergothioneine acts as an inhibitor in the formation of hydroxy radicals.

BHA and BHT were also examined for inhibition of DNA damage by reduced dyes (Table 3.10). They were not suitable for use as antioxidants in this assay as they were genotoxic in their own right.

TABLE 3.10 Effect of chemical antioxidants on survival of *E. coli* WP2 and CM871

Antioxidant final concentration (mM)	Coefficient of Survival			
	BHA		BHT	
	Control	+ Reduced Amaranth (5mM)	Control	+ Reduced Amaranth (5mM)
0.00	1.0 ± 0.03	0.12 ± 0.03	1.0 ± 0.03	0.14 ± 0.03
0.01	0.45 ± 0.14	0.11 ± 0.06	0.19 ± 0.07	0.10 ± 0.04
0.05	0.37 ± 0.02	0.13 ± 0.07	0.1 ± 0.04	0.02 ± 0.06
0.10	0.31 ± 0.15	0.11 ± 0.06	0.13 ± 0.09	0.02 ± 0.08
0.50	0.21 ± 0.16	0.09 ± 0.01	0.12 ± 0.03	0.01 ± 0.08
1.00	0.16 ± 0.17	0.06 ± 0.02	0.12 ± 0.02	0.02 ± 0.04
5.00	0.11 ± 0.10	0.04 ± 0.01	Cytotoxic#	0.02 ± 0.03
10.00	0.07 ± 0.12	0.03 ± 0.02	Cytotoxic#	Cytotoxic#

Mean ± SD of 4 independent experiments

CS = $\frac{\%\ \text{survivors of repair deficient strain}}{\%\ \text{survivors of repair proficient strain}}$

Cytotoxic: Excessive wild type kill

3.5.8 Genotoxicity of superoxide anions in the differential killing assay

From my studies it was concluded that there was a requirement for oxygen before reduced dyes exhibited a positive effect in the differential kill assay (Section 3.5.1). It was therefore important to determine whether oxygen was involved in the mechanism behind the genotoxicity of these reduced azo dyes. Initially, it was necessary to determine whether oxygen radical species had the potential to cause damage to the *E. coli* species used in the differential kill assay.

Hydroxyl radicals were generated in a model system containing xanthine plus xanthine oxidase (to generate superoxide radicals and hydrogen peroxide), salicylate and traces of Fe^{2+} and Fe^{3+}. Formation of diphenolic products from salicylate in this system was inhibited by superoxide dismutase, catalase and scavengers of OH' radicals. Millimolar concentrations of EDTA increased reactive oxygen species generation, whereas similar concentrations of desferrioxamine inhibited hydroxyl radical generation (Section 3.7).

Xanthine oxidase (EC1.1.3.22) produced superoxide radicals during oxidation of xanthine to uric acid. Superoxide radicals were detected by reduction of nitroblue tetrazolium. The reduction of NBT to formazan (an insoluble precipitate) was inhibited by the addition of superoxide dismutase, while inactivated superoxide dismutase showed no inhibitory effect (Section 3.7).

The superoxide radicals generated were found to produce a positive response in the differential kill assay using tester strains *E. coli* WP2 & CM871. The presence of superoxide dismutase resulted in a decrease in genotoxicity, while deactivated superoxide dismutase (dSOD) had no effect on genotoxicity. Superoxide radicals produced no response in the tester strains *E. coli* AB1157 & BH20. Table 3.11 summarises results obtained. There was no effect under strict anaerobic conditions, showing molecular oxygen was essential for genotoxicity.

TABLE 3.11 Genotoxicity exhibited by xanthine/xanthine oxidase-generated superoxide anion

Differential kill assay tester pairs	Coefficient of Survival[a] Superoxide Generating System			
	Control	+ superoxide dismutase[b]	+ dSOD[c]	Anaerobic
E. coli WP2 & CM871	0.28± 0.03	0.91± 0.01	0.34± 0.02	0.89± 0.02
E. coli AB1157 & BH20	0.93± 0.02	0.93± 0.03	0.92± 0.01	1.00± 0.01

Mean ± SD of 4 independent experiments

a CS = $\frac{\text{\% survivors of repair deficient strain}}{\text{\% survivors of repair proficient strain}}$

b SOD Superoxide dismutase (1mg/ml)

c dSOD deactivated superoxide dismutase (1mg/ml)

Genotoxicity of hydrogen peroxide was also examined in the differential kill assay using the two tester pairs, *E. coli* WP2 & CM871 and *E. coli* AB1157 & BH20. Hydrogen peroxide produced a positive response in the tester pair E. coli WP2 & CM871 at concentrations greater than 0.01mM. Hydrogen peroxide was not genotoxic in the tester pair *E. coli* AB1157 & BH20. This supports the findings of Boiteux and Huisman (1989) who found the *fpg*$^-$ mutant *E. coli* BH20 did not exhibit increased sensitivity to H_2O_2 (Table 3.12).

Based on these results there was a suggestion that hydrogen peroxide, hydroxyl radicals and superoxide radicals were in some way involved either directly or indirectly in genotoxicity of reduced azo dyes. It was also apparent from azo reduction and end-product studies that the mechanism behind genotoxicity of these azo dyes was based upon the ease by which a dye was reduced and its transformation into an ortho-hydroxy amine.

Initially it was determined whether hydrogen peroxide was produced on reduction of azo dyes or by their predicted end-products. It was also important to determine whether hydrogen peroxide was responsible for the genotoxicity exhibited by these compounds. Intracellular catalase was inhibited to determine whether this would render *E. coli* cells equally susceptible to damage induced by reduced dyes. The *E. coli* tester strains, WP2 & CM871 were preincubated with 12mM aminotriazole for 1 hour to deactivate completely intracellular catalase before addition of the test compound. Loss of catalase activity was determined using the catalase test. The preincubation of the tester strains with aminotriazole resulted in an increase in genotoxicity exhibited by reduced sunset yellow (Figure 3.10a), while the data in Figure 3.10b indicate that *E. coli* CM871 became more susceptible to damage by 1-amino-2-naphthol-6-sulphonate after pre-incubation with aminotriazole. Results clearly demonstrate that intracellular catalase provided *E. coli* tester strains WP2 & CM871 protection against damage by reduced dyes and their predicted end-products. Addition of catalase to the system provided complete protection against genotoxicity due to reduced sunset yellow and its predicted end-product (Figure 3.10a&b).

TABLE 3.12 Genotoxicity exhibited by hydrogen peroxide

Hydrogen peroxide concentration (mM)	Coefficient of Survival[a] Differential kill assay tester pairs			
	E. coli WP2 & CM871		*E. coli* AB1157 & BH20	
	Hydrogen Peroxide	Hydrogen Peroxide + Catalase[b]	Hydrogen Peroxide	Hydrogen Peroxide + Catalase[b]
0	1.0 ± 0.01	1.0 ± 0.05	1.0 ± 0.01	1.0 ± 0.032
0.001	1.0 ± 0.08	1.0 ± 0.02	1.0 ± 0.02	1.0 ± 0.071
0.01	0.38 ± 0.05	1.0 ± 0.01	1.0 ± 0.01	1.0 ± 0.045
0.1	0.21 ± 0.03	1.0 ± 0.09	0.99 ± 0.06	1.0 ± 0.003
1.0	0.09 ± 0.09	1.0 ± 0.07	0.99 ± 0.006	1.0 ± 0.022
5.0	0.02 ± 0.10	0.95 ± 0.04	0.99 ± 0.01	1.0 ± 0.009
10.0	0.0 ± 0.13	0.97 ± 0.06	0.99 ± 0.005	1.0 ± 0.001

Mean ± SD of 4 independent experiments

a CS = $\frac{\text{\% survivors of repair deficient strain}}{\text{\% survivors of repair proficient strain}}$

b Catalase 1mg/ml

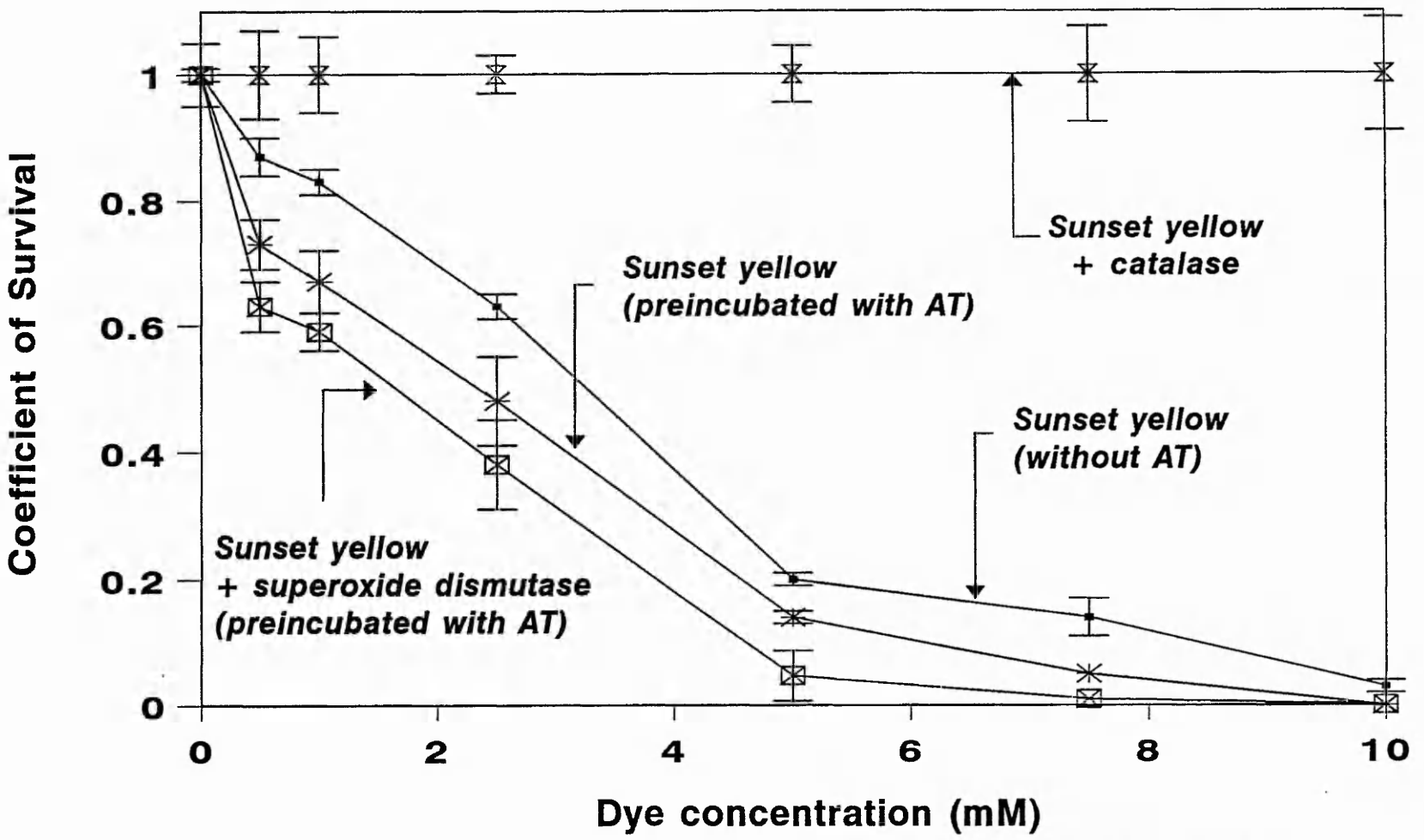


Figure 3.10a Genotoxicity exhibited by sunset yellow after reduction by *E. faecalis: Tester strains *E. coli* WP2 & CM871 preincubated with aminotriazole (12mM for 1 hour)**

*** Washed cell suspension**

Data are means +/- SD of 4 independent experiments

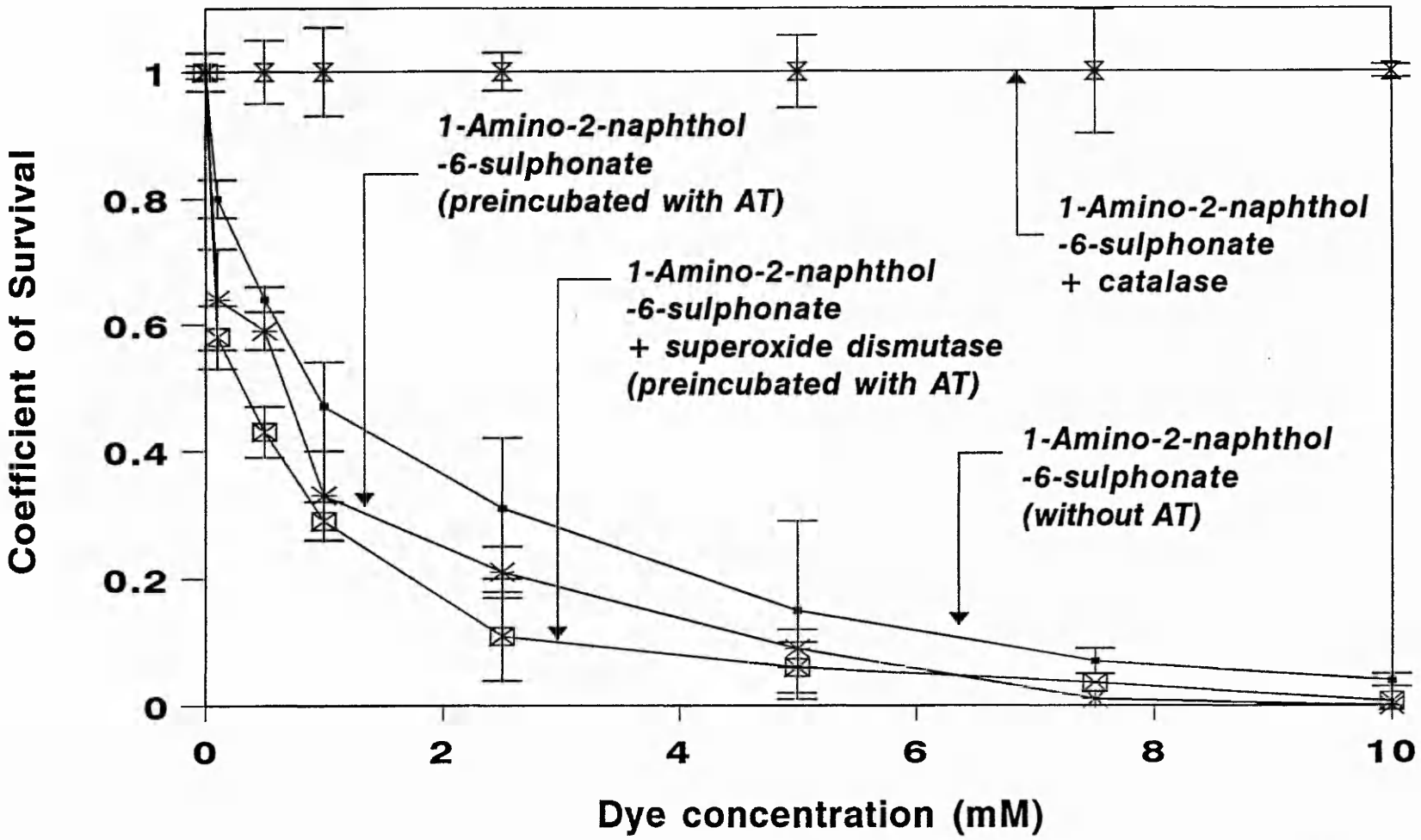


Figure 3.10b Genotoxicity exhibited by 1-amino-2-naphthol -6-sulphonate: Tester strains *E. coli* WP2 & CM871 preincubated with aminotriazole (12mM for 1 hour)

Data are means +/- SD of 4 independent experiments

The data suggest that since hydrogen peroxide is the prime target for catalase, it is being generated from reduced dyes and aminonaphthols. Hydrogen peroxide may be generated externally by reduced dyes and subsequently penetrate the cells to induce DNA damage. It is also possible that the reduced dyes and aminonaphthols enter the cells and generate hydrogen peroxide internally. The addition of extracellular catalase (1mg/ml), however, completely prevented DNA damage implying that at some stage in the reaction hydrogen peroxide must leak out of the bacterial cell. SOD (1mg/ml) had no protective effect. The protection of *E. coli* by intracellular catalase underscores the importance of catalase in the protection of DNA from oxidant attack.

Further experiments were performed to confirm the involvement of active oxygen species in genotoxicity of reduced azo dyes. The following compounds were examined for their ability to prevent induced DNA damage in *E. coli* by reduced azo dyes:

1. 1-10-phenanthroline, an iron chelator which prevents the formation of hydroxy radicals from hydrogen peroxide and DNA-bound iron by the Fenton reaction;
2. desferrioxamine, a powerful chelator of iron and inhibitor of hydroxy radical generation;
3. potassium iodide, an hydroxyl radical scavenger.

1-10-Phenanthroline, desferrioxamine and potassium iodide, all significantly protected *E. coli* from damage by both reduced azo dyes and their predicted end-products (Table 3.13). Iron ions bound to desferrioxamine are usually poorly active, or inactive, in promoting iron dependent radical reactions. The chelating agent EDTA commonly used does not prevent the reaction of iron ions with H_2O_2 or with oxygen radicals. Copper-EDTA chelates are usually less active than free copper ions in radical reactions, whereas chelates of EDTA with iron still react with hydrogen peroxide or superoxide radicals.

Since hydroxyl radicals react indiscriminately and have diffusion radii of 2.3nm (Schraufstatter *et al.*, 1988), one may assume that the hydroxyl radical scavengers never reached the site of hydroxyl radical attack on the DNA. Since the rate constant for the reaction of various hydroxyl scavengers is of the same order of magnitude as that of

hydroxyl radicals with deoxyribose and since nucleotides are present in molar concentrations in the nucleosome, it was not surprising that hydroxyl radical scavengers could not compete efficiently in this reaction.

Potassium iodide, an hydroxyl radical scavenger significantly inhibited induced DNA damage by reduced azo dye or aminonaphthol in the *E. coli* pair WP2 & CM871. This suggests that hydroxyl radicals generated by a metal catalysed Fenton-type reaction contribute to reduced azo dye-induced damage in this strain of *E. coli*. Table 3.13 shows the influence of oxidant scavengers on the formation of damage induced by reduced azo dyes in the *E. coli* pair WP2 & CM871.

TABLE 3.13 Influence of oxidant scavengers on genotoxicity of reduced azo dyes and their predicted end-products

Tester strains *E. coli* WP2 and CM871

Scavenged Species	Scavenger	% Inhibition of cell death			
		Reduced sunset yellow (5mM)[a]	1-amino-2-naphthol,6-sulphonate (2.5mM)	Reduced amaranth (5mM)	1-amino-2-naphthol-3,6 sulphonate (1.0mM)
Superoxide radicals	Superoxide dismutase	5	4	6	5
Hydrogen	Catalase	100	100	100	100
peroxide	Aminotriazole	No inhibition	No inhibition	No inhibition	No inhibition
Hydroxyl	Mannitol	No inhibition	No inhibition	No inhibition	No inhibition
radical	Potassium iodide	11	15	14	12

a Concentrations used were based on the minimum concentration which elicited a positive response in the differential kill assay based on tester strains *E. coli* WP2 and CM871
b DFO Desferrioxamine

TABLE 3.13 Influence of oxidant scavengers on genotoxicity of reduced azo dyes and their predicted end-products (Continued)

Tester strains *E. coli* WP2 and CM871

Scavenged Species	Scavenger	% Inhibition of cell death			
		Reduced sunset yellow (5mM) [a]	1-amino-2-naphthol,6-sulphonate (2.5mM)	Reduced amaranth (5mM)	1-amino-2-naphthol-3,6 sulphonate (1.0mM)
Iron chelator	DFO[b] (15min incubation)	No inhibition	No inhibition	No inhibition	No inhibition
	DFO[b] (overnight incubation)	90	80	75	70
	Phenanthroline	60	65	65	55
	EDTA	No inhibition	No inhibition	No inhibition	No inhibition
Lipid peroxides	Ascorbic acid	100	100	100	100
	α-Tocopherol (overnight)	64	61	68	63
	Retinoids	58	69	59	55
	BHA/BHT	No inhibition	No inhibition	No inhibition	No inhibition
	l-Ergothionine	100	100	100	100
	Chlorophyllin	87	82	83	87

a Concentrations used were based on the minimum concentration which elicited a positive response in the differential kill assay based on tester strains *E. coli* WP2 and CM871
b DFO Desferrioxamine

3.6 SALMONELLA MUTAGENICITY TEST

3.6.1 Tests without azo reduction

When samples of amaranth, carmoisine, sunset yellow were tested directly in the standard plate assay using *S.typhimurium* strains TA102 and TA104, at concentrations ranging from 0.01 to 10 μg/plate no detectable mutagenic activity was observed in the absence or presence of rat liver S9.

Azobenzene and its derivative, *p*-phenylazobenzene, were both non-mutagenic at the concentrations studied (0.01 -10μg/plate). None of the phenyl-naphthol dyes (sudan I, orange II, orange G and orange I) or the naphthalene-naphthol dyes (bordeau red, ponceau 6R and Eriochrome blue black) exhibited mutagenicity in *S. typhimurium* TA102 or TA104 at the concentrations studied (0.01 - 10μg/plate). None of the unreduced dyes were activated in the presence of S9.

3.6.2 Tests with azo reduction

The activity of the azo dyes amaranth, carmoisine and sunset yellow was enhanced by azo reduction by *E. faecalis*. This indicates that reduction of the azo bond is an important step in the activation of these dyes to mutagens. The presence of S9 enhanced mutagenicity of the reduced azo dyes (Table 3.14a & b). This response was only demonstrated when *S. typhimurium* TA102 or TA104 were used in the assay. Mutagenicity was not expressed when TA98 and TA100 were used as the tester strains.

The phenyl-naphthol dyes, sudan I, and orange II were mutagenic after azo reduction by *E. faecalis* at concentrations of 0.5μg/plate and above. Eriochrome blue black, a naphthalene-naphthol dye was also mutagenic at concentrations of 0.5μg/plate and above after reduction by *E. faecalis* (Table 3.15a & b). The presence of S9 had no significant effect on the activity of these compounds.

TABLE 3.14a *Salmonella* microsome assay with TA102: tests with azo reduction

Reduced azo dye concentration (μg/plate)	No. of his$^+$ revertant colonies /plate					
	Amaranth	Amaranth + S9	Sunset Yellow	Sunset Yellow+S9	Carmoisine	Carmoisine+S9
0.0	240 ± 16	232 ± 18	235 ± 16	232 ± 18	240 ± 16	232 ± 18
0.01	222 ± 17	419 ± 17	184 ± 7	337 ± 54	210 ± 16	256 ± 18
0.05	448 ± 16	461 ± 16	252 ± 48	389 ± 41	221 ± 6	266 ± 10
0.1	454 ± 15	553 ± 20	275 ± 18	464 ± 24	245 ± 4	289 ± 10
0.25	490 ± 27	534 ± 14	318 ± 39	501 ± 21	298 ± 13	345 ± 30
0.5	566 ± 17	583 ± 30	322 ± 31	483 ± 28	297 ± 17	368 ± 14
0.75	470 ± 18	**940 ± 10***	**774 ± 37***	**752 ± 15***	305 ± 12	398 ± 20
1.0	**5870 ± 257***	7138 ± 96	5984 ± 150	5987 ± 56	378 ± 16	401 ± 60
2.0	716 ± 180	561 ± 38	555 ± 60	678 ± 76	389 ± 18	412 ± 73
5.0	533 ± 160	581 ± 12	540 ± 70	598 ± 38	401 ± 5	398 ± 87
10.0	542 ± 130	589 ± 24	567 ± 70	545 ± 10	422 ± 17	412 ± 45

Mean ± SD of 4 independent experiments

* Threshold over which compounds were considered mutagenic

Figures in bold indicate significant differences (ANOVA) $P<0.05$, compared with appropriate control groups

TABLE 3.14b *Salmonella* microsome assay with TA104: tests with azo reduction

Reduced azo dye concentration (μg/plate)	No. of his$^+$ revertant colonies /plate					
	Amaranth	Amaranth + S9	Sunset Yellow	Sunset Yellow+S9	Carmoisine	Carmoisine+S9
0.0	245 ± 18	242 ± 12	245 ± 18	245 ± 12	257 ± 18	245 ± 12
0.01	306 ± 18	369 ± 17	220 ± 13	284 ± 12	245 ± 19	256 ± 47
0.05	411 ± 16	584 ±29	212 ± 12	301 ± 42	249 ± 15	278 ± 33
0.1	448 ± 13	623 ± 41	363 ± 17	376 ± 31	268 ± 18	794 ± 32
0.25	576 ± 15	677 ± 54	587 ± 15	468 ± 18	264 ± 12	305 ± 48
0.5	777 ± 16	798 ± 68	589 ± 10	645 ± 30	289 ± 15	346 ± 40
0.75	816 ± 33	960 ± 84	659 ± 5	788 ± 41	305 ± 12	378 ± 63
1.0	**912 ± 10***	**970 ± 92***	663 ± 13	**921 ± 55***	345 ± 12	389 ± 23
2.0	1005 ± 86	982 ± 81	671 ± 63	956 ± 70	398 ± 10	401 ± 38
5.0	1011 ± 99	999 ± 31	**892 ± 83***	947 ± 84	401 ± 12	401 ± 74
10.0	1101 ± 55	1316 ± 101	1145 ± 87	1121 ± 93	456 ± 13	501 ± 34

Mean ± SD of 4 independent experiments

* Threshold over which compounds were considered mutagenic

Figures in bold indicate significant differences (ANOVA) $P<0.05$, compared with appropriate control groups

TABLE 3.15a *Salmonella* microsome assay with TA102: tests with azo reduction

Reduced azo dye concentration	No. of his$^+$ revertant colonies /plate					
(µg/plate)	EBB	EBB + S9	Sudan I	Sudan I+S9	Orange II	Orange II+S9
0.0	215 ± 12	247 ± 24	215 ± 12	247 ± 24	215 ± 12	247 ± 24
0.5	**845 ± 14***	**946 ± 12***	**1020 ± 34***	**1100 ± 13***	**745 ± 33***	**810 ± 32***
1.0	1400 ± 77	1650 ± 56	2300 ± 76	2500 ± 89	1089 ± 29	1300 ± 78
2.5	4000 ± 96	4300 ± 78	5100 ± 56	5300 ± 120	3420 ± 78	3500 ± 39
50.0	No lawn	No lawn	No lawn	No lawn	No lawn	No lawn

TABLE 3.15b *Salmonella* microsome assay with TA104: tests with azo reduction

Reduced azo dye concentration	No. of his$^+$ revertant colonies /plate					
(µg/plate)	EBB	EBB + S9	Sudan I	Sudan I+S9	Orange II	Orange II+S9
0.0	239 ± 15	268 ± 23	239 ± 15	268 ± 23	239 ± 15	268 ± 23
0.5	**1040 ± 22***	**1400± 19***	**1900 ± 77***	**2100 ± 325***	**910 ± 80***	**1030± 67***
1.0	2100 ± 55	2500 ± 54	3900 ± 45	4100 ± 247	1980 ± 100	2101 ± 56
2.5	4700 ± 35	5100 ± 36	5800 ± 45	5190 ± 234	4120 ± 87	4300 ± 40
50.0	No lawn	No lawn	No lawn	No lawn	No lawn	No lawn

Mean ± SD of 4 independent experiments
* Threshold over which compounds were considered mutagenic
Figures in bold indicate significant differences (ANOVA) $P<0.05$, compared with appropriate control groups

When amaranth and sunset yellow were tested in the modified preincubation protocol with FMN in the presence of rat liver S9, no significant mutagenic activity was detected (Table 3.16a & b). Negative results in this assay may be due to the presence of the free unreduced azo compound which can interfere with the ability to detect the mutagenic activity of the reduced azo dye (Section 1.5.1.2).

The mutagenicity of reduction products can be tested using the chemical reduction and extraction methods as described by Prival and Mitchell (1992). This method uses dithionite, a mild reducing agent. However, it has not been demonstrated that dithionite does not cause chemical changes other than the type of azo reduction that would occur in the intestine *in vivo*. Thus anaerobic reduction of the azo dye by an intestinal microorganism may be the better technique.

TABLE 3.16a *Salmonella* microsome assay with TA102:

Tests with FMN azo reduction

Azo dye concentration	No. of his^+ revertant colonies /plate		
(μg/plate)	Amaranth	Sunset Yellow	Carmoisine
0.0	257 ± 21	257 ±21	257±21
0.01	250 ± 15	241 ±18	259 ± 10
0.05	261 ± 18	257 ± 21	247 ± 8
0.1	270 ± 24	237 ± 31	221 ± 13
0.25	278 ± 12	253 ± 15	213 ± 32
0.5	291 ± 15	261 ± 32	235 ± 20
0.75	304 ± 12	277 ± 32	247 ± 14
1.0	348 ± 19	285 ± 10	250 ± 25
2.0	364 ± 21	295 ± 32	258 ± 21
5.0	378 ± 32	301 ± 12	275 ± 24
10.0	448 ± 13	325 ± 5	276 ± 12

Mean ± SD of 4 independent experiments
* Threshold over which compounds were considered mutagenic
Figures in bold indicate significant differences (ANOVA) $P<0.05$, compared with appropriate control groups

TABLE 3.16b *Salmonella* microsome assay with TA104: Tests with FMN azo reduction

Azo dye concentration	No. of his$^+$ revertant colonies /plate		
(μg/plate)	Amaranth	Sunset Yellow	Carmoisine
0.0	231 ± 12	231 ± 12	231 ± 12
0.01	248 ± 19	237 ± 25	218 ± 15
0.05	251 ± 21	243 ± 14	213 ± 17
0.1	266 ± 35	249 ± 12	208 ± 14
0.25	289 ± 15	244 ± 16	214 ± 15
0.5	301 ± 16	257 ± 19	213 ± 14
0.75	336 ± 21	255 ± 9	215 ± 12
1.0	357 ± 12	261 ± 21	241 ± 19
2.0	371 ± 13	278 ± 31	256 ± 12
5.0	389 ± 16	289 ± 14	266 ± 15
10.0	405 ± 13	311 ± 15	289 ± 13

Mean ± SD of 4 independent experiments
* Threshold over which compounds were considered mutagenic
Figures in bold indicate significant differences (ANOVA) $P<0.05$, compared with appropriate control groups

3.6.3 Mutagenicity of predicted end-products

1-Amino-2-naphthol exhibited a positive mutagenic response to TA102 and TA104 at concentrations greater than 0.01μg/plate (Figure 3.11). 1-Amino-2-naphthol-4-sulphonate, predicted as being the second azo reduction product of EBB was also tested for genotoxicity. It was mutagenic in strains *S. typhimurium* TA102 and TA104. The presence of S9 in the reaction mix had no significant effect on mutagenicity of these compounds.

The mutagenic potency of 1-amino-2-naphthol-3,6-disulphonate and 1-amino-2-naphthol-6-sulphonate within the concentration range 0.01-10μg/plate was also assessed using *S.typhimurim* TA102 and TA104 in the 30min preincubation mutagenicity assay. As before uninduced rat liver S9 preparations were employed for metabolic activation. Under these conditions both 1-amino-2-naphthol-6-sulphonate and 1-amino-2-naphthol-3,6-sulphonate gave a doubling of the spontaneous reversion rate (Figure 3.12). At 5μmol/plate the number of revertants for the predicted end-product of amaranth, 1-amino-2-naphthol-3,6-sulphonate were 659 ± 15, 1936 ± 32 for control and S9 activated respectively using *S.typhimurium* TA102. At 5μmol/plate the number of revertants for 1-amino-2-naphthol-6-sulphonate were 437 ± 89, 1329 ± 67 for control and S9 activated respectively.

To identify the mechanism behind mutagenicity of the aminonaphthols, they were examined in the presence of specific inhibitors, as determined from the differential killing assays. The mutagenicity of these compounds in both the presence and absence of S9 was inhibited by extracellular catalase (1mg/ml). Mutagenicity of 1-amino-2-naphthol-3,6-sulphonate was reduced by 37%. Mutagenicity was only inhibited by less than 1% when incubated with superoxide dismutase (1.0mg/ml). These results compared well with those obtained using *S. typhimurium* TA104. The inhibitor concentrations used gave maximum inhibition and yet showed no signs of cytotoxicity, as was evidenced by maintenance of background lawn.

Superoxide anions were generated as for the differential killing assay. These were found to be mutagenic in *S. typhimurium* TA102 and TA104 (Table 3.17).

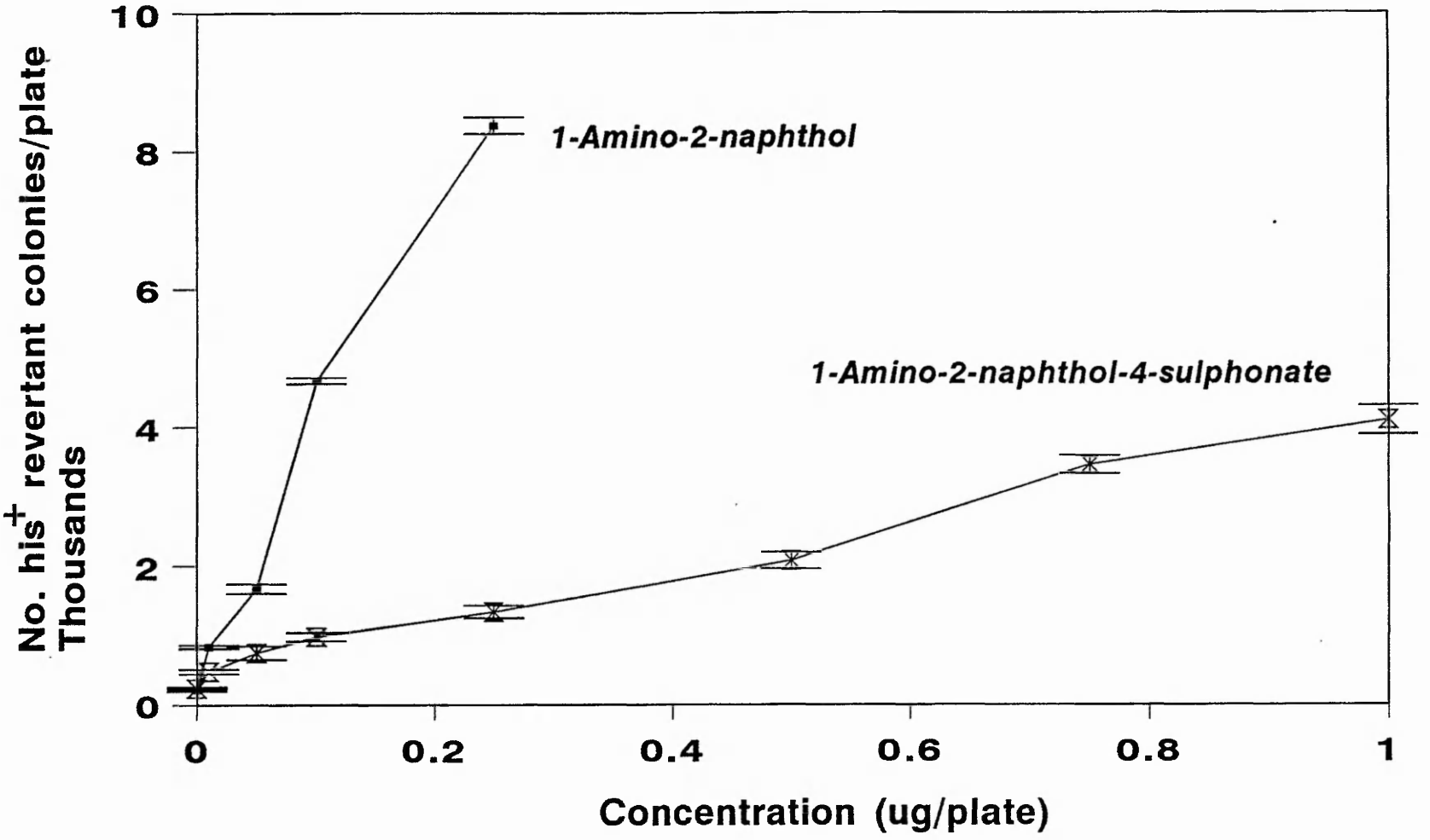


Figure 3.11 Mutagenicity exhibited by the predicted end-products of erriochrome blue black: Tester strain *S. typhimurium* TA102

Data are means +/- SD of 4 independent experiments

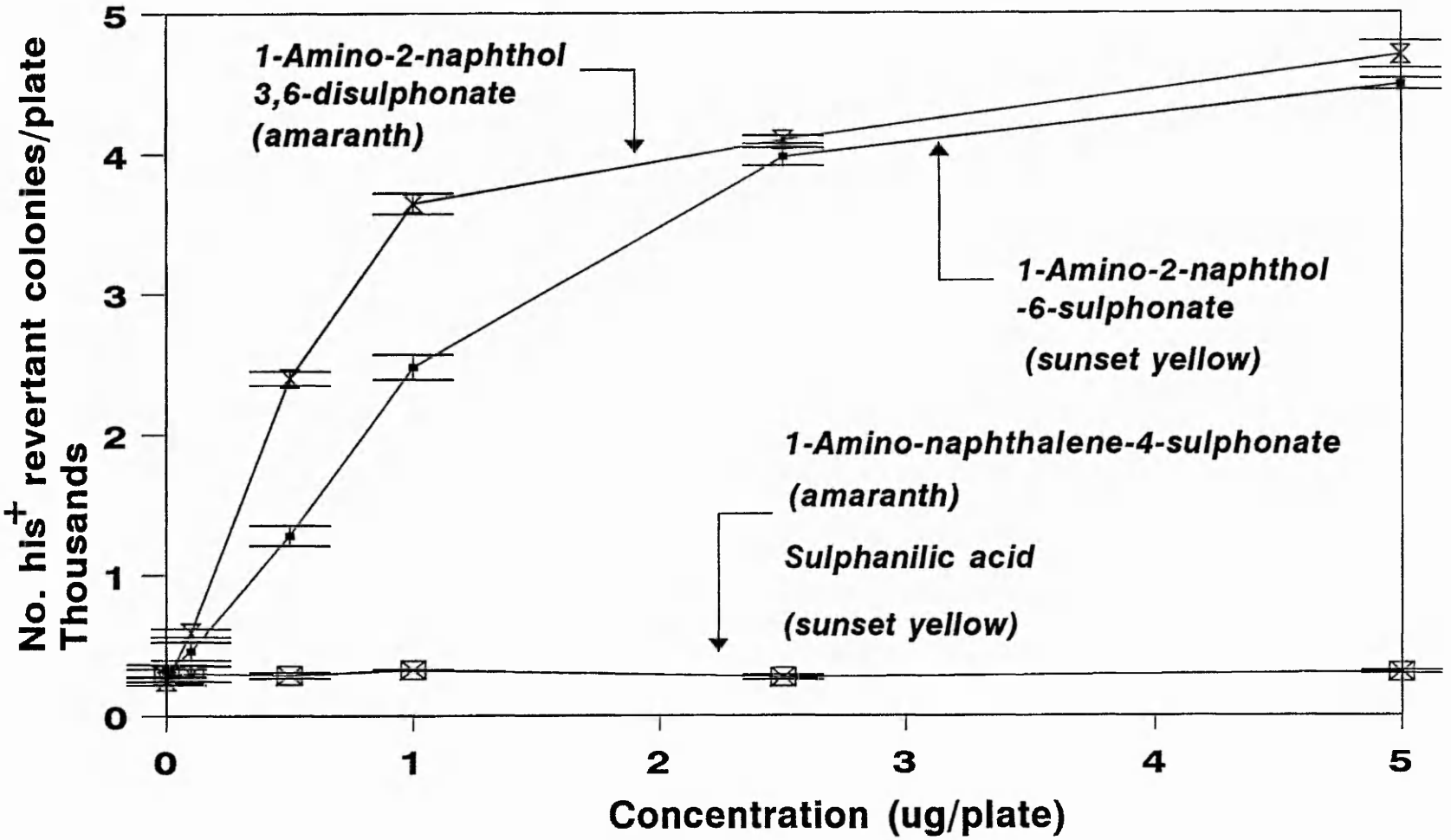


Figure 3.12 Mutagenicity exhibited by the predicted end-products of amaranth and sunset yellow: Tester strain *S. typhimurium* TA102

Data are means +/- SD of 4 independent experiments

TABLE 3.17 Mutagenicity exhibited by xanthine/xanthine oxidase-generated superoxide anion

Detector strain	No. of his^+ revertant colonies /plate (minus spontaneous) Superoxide Generating System		
	Control	+ superoxide dismutase[a]	+ dSOD[b]
S. typhimurium TA102	355 ± 21	121 ± 65	291 ± 56
S. typhimurium TA102 + S9	431 ± 81	219 ± 44	312 ± 72
S. typhimurium TA104	410 ± 78	147 ± 45	371 ± 32
S. typhimurium TA104 + S9	437 ± 52	165 ± 81	431 ± 31

Mean ± SD of 4 independent experiments
a Superoxide dismutase (1mg/ml)
b dSOD deactivated superoxide dismutase (1mg/ml)

3.7 DETECTION OF SUPEROXIDE ANION RADICAL

The nitroblue tetrazolium and cytochrome C assays were based on the ability of an indicator molecule to scavenge superoxide radicals. The reaction was followed spectrophotometrically in terms of the accumulations of blue formazan (NBT assay) or ferrocytochrome C (cytochrome C assay). As there are agents other than superoxide radicals that can cause these reductions, superoxide dismutase was used to distinguish reactions due to the presence of superoxide radical from those due to other reactants. SOD, by intercepting superoxide radicals, has the effect of specifically inhibiting reactions involving this radical. The reaction of NBT with superoxide radicals is very complex and is discussed in greater detail in the introduction. Blue formazan, the product of the reaction of NBT with superoxide radicals is only sparingly soluble in water, its precipitation was avoided by keeping absorbance changes fairly low.

Generation of superoxide radicals was observed for reduced dyes and their predicted end-products, but not for unreduced dyes (Table 3.18). In studies examining the production of superoxide radicals from chemically reduced azo dyes, it was found that sodium dithionite was readily oxidised in solution with simultaneous production of superoxide radicals. Thus stannous chloride was used as the chemical reducing agent as it did not produce superoxide radicals (Figure 3.13). Chemically reduced azo dyes generated a higher concentration of superoxide radical than *E. faecalis*-reduced dyes, in both the NBT and cytochrome C assay. The concentration of superoxide radical produced from azo dyes reduced by *B. thetaiotaomicron* was consistently lower than that produced by *E. faecalis*. As rate of reduction for *B. thetaiotaomicron* is lower than the rate of reduction of *E. faecalis,* superoxide radicals may have a greater chance to disperse when *B. thetaiotaomicron* is used as the reducing agent. There was a good correlation between results obtained from the NBT assay and the cytochrome C assay.

The presence of superoxide dismutase in the reaction mix resulted in a decrease in absorbance at 540nm (NBT assay), while inactivated SOD showed no inhibition (Figure 3.14). In compounds tested the presence of an hydroxy group ortho to the amino group was essential for generation of superoxide radicals (Table 3.18).

TABLE 3.18 Generation of superoxide radicals from azo dyes

Azo Dye	Rate of superoxide radical production/μmol compound/min					
	Nitro Blue Tetrazolium Assay			Cytochrome C Assay		
	Azo reduction by			Azo reduction by		
	E. faecalis	*B. thetaiotaomicron*	Stannous chloride	*E. faecalis*	*B. thetaiotaomicron*	Stannous chloride
Amaranth	0.023 ± 0.002	0.016 ± 0.01	0.062 ± 0.002	0.13 ± 0.003	0.11 ± 0.01	0.34 ± 0.02
Sunset yellow	0.026 ± 0.022	0.014 ± 0.001	0.055 ± 0.003	0.12 ± 0.001	0.09 ± 0.02	0.29 ± 0.05
Carmoisine	0.019 ± 0.001	0.008 ± 0.006	0.02 ± 0.002	0.07 ± 0.007	0.04 ± 0.01	0.14 ± 0.01

Mean ± SD of 4 independent experiments

TABLE 3.18 Generation of superoxide radicals from predicted end-products (Continued)

Predicted End-product	Rate of superoxide radical production/μmol compound/min	
	Nitro Blue Tetrazolium Assay	Cytochrome C Assay
1-Amino-2-naphthol	1.07 ± 0.03	0.18 ± 0.04
1-Amino-2-naphthol-4-sulphonate	0.022 ± 0.002	0.05 ± 0.01
1-Amino-2-naphthol-6-sulphonate	0.041 ± 0.01	0.21 ± 0.02
1-Amino-2-naphthol-3,6-disulphonate	0.039 ± 0.03	0.24 + 0.02
1-Amino-naphthalene-4-sulphonate	$1.34 \times 10^{-4} \pm 0.0$	0.01 ± 0.01

Mean ± SD of 4 independent experiments

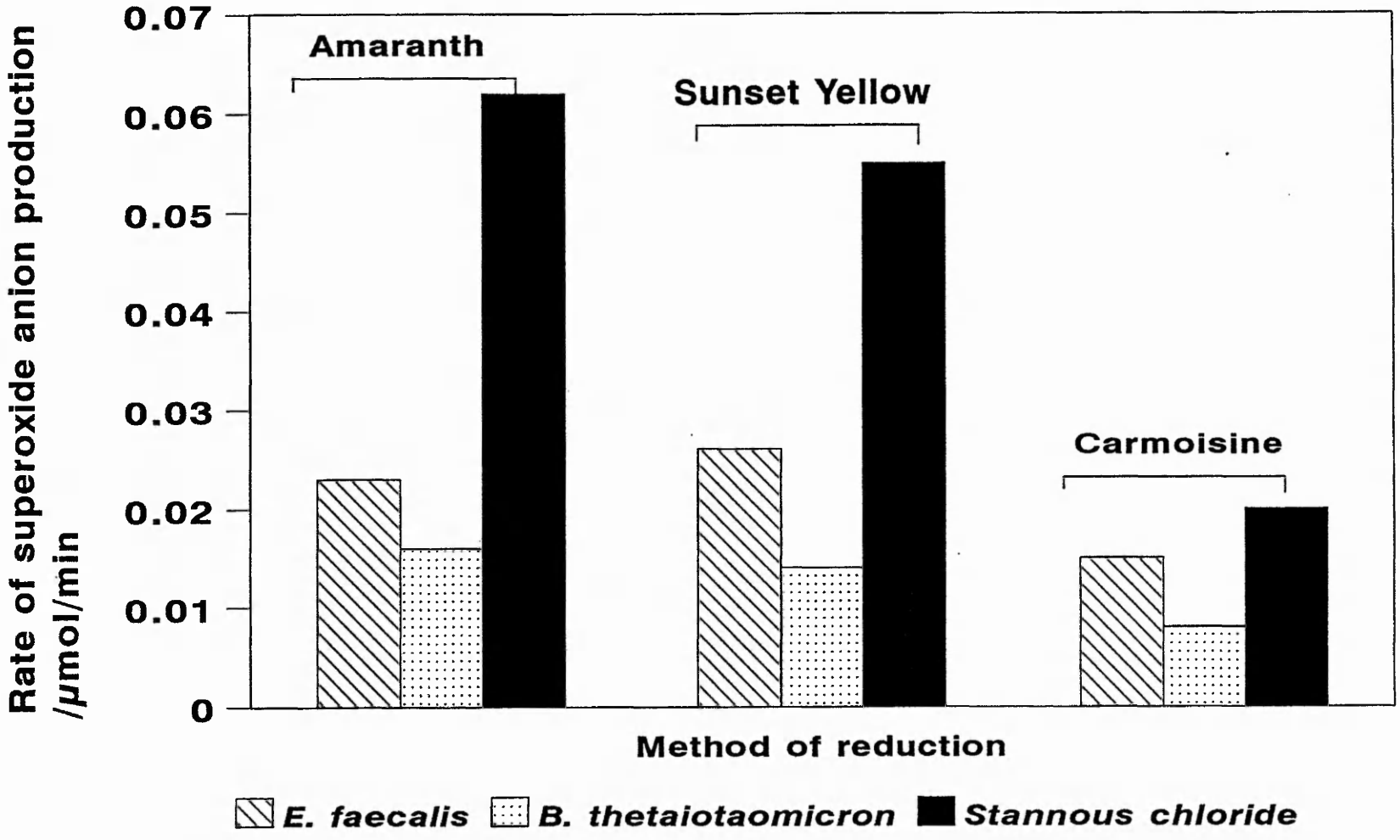


Figure 3.13 Effect of method of azo reduction on generation of superoxide radicals: Nitroblue tetrazolium assay

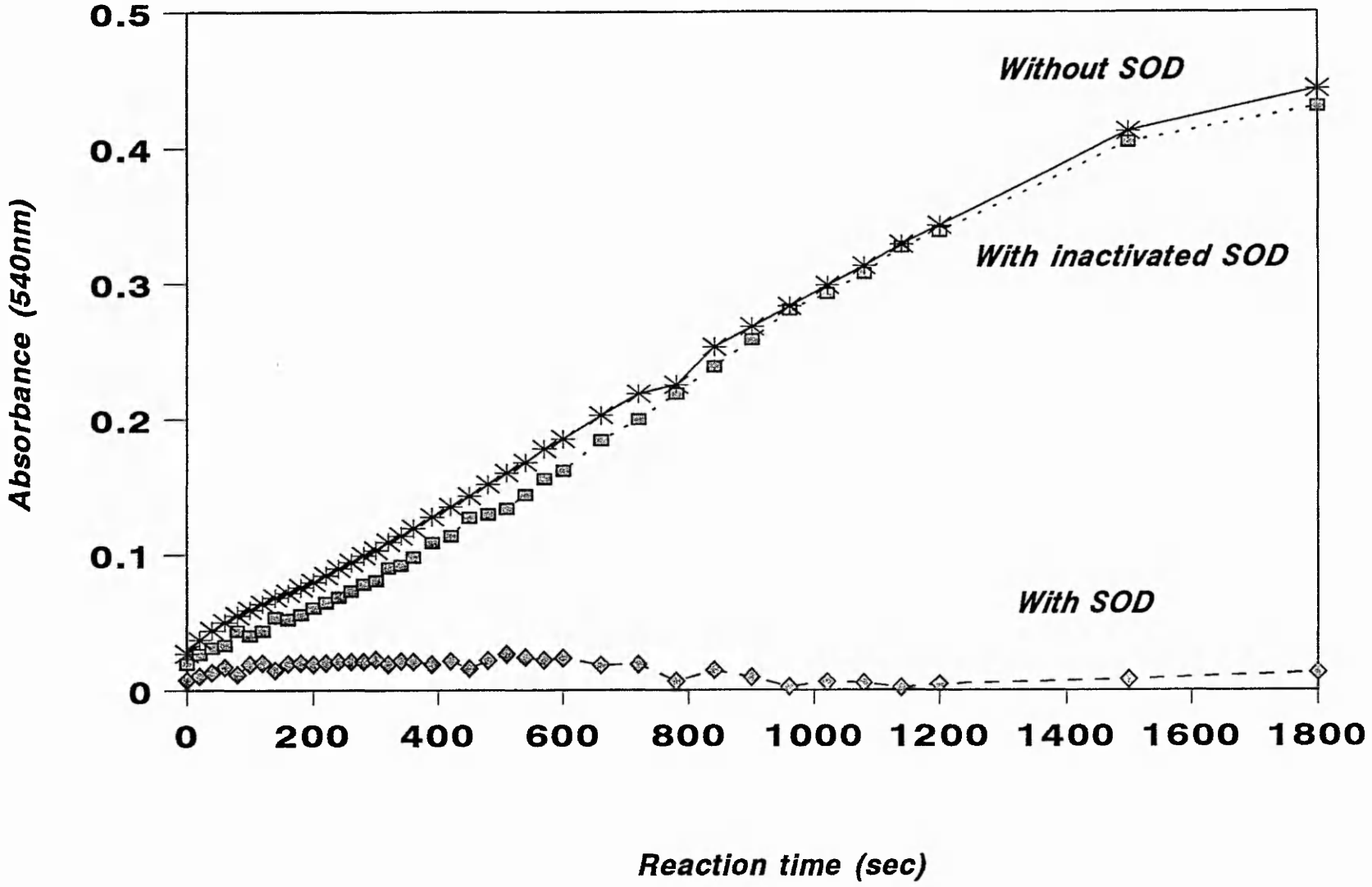


Figure 3.14 Generation of superoxide radicals from E. faecalis-reduced sunset yellow: NBT assay

Rate of superoxide production from 1-amino-2-naphthol (NBT assay) was 1.07±0.03 /μmol compound/min. While negligible superoxide radical was produced by 1-amino-naphthalene-4-sulphonic acid ($1.34x10^{-4}$/μmol compound/min). The presence of sulphonate groups on the naphthol moiety resulted in a decrease in the amount of superoxide radicals generated. The rate of superoxide production for 1-amino-2-naphthol-6-sulphonate and 1-amino-2-naphthol-3,6-disulphonate was 0.041±0.01 and 0.039±0.03 /μmol compound/min respectively.

Results were further confirmed by an ability to reduce cytochrome c. The presence of SOD resulted in a decrease in activity, while deactivated SOD had no effect on detection of superoxide radicals. As for the NBT assay, a hydroxy group ortho to the amino group stimulated the generation of superoxide radicals, 0.18±0.04 /μmol compound/min for 1-amino-2-naphthol. 1-amino-naphthalene-4-sulphonate produced negligible detectable superoxide radical (0.01±0.01/μmol compound/min). Increased substitution by a sulphonate group resulted in a decrease in detection of superoxide radicals (Table 3.18).

3.8 DETECTION OF HYDROGEN PEROXIDE

Production of H_2O_2 was followed using the method of Colowick and colleagues (1984) with slight modification. The reaction depended upon the peroxidase-catalysed oxidation of a suitable substrate to yield a coloured product which was detected spectrophotometrically. H_2O_2, a potent oxidising agent coupled oxidatively with 4-aminoantipyrene and phenol to yield a quinoneimine dye with a maximum absorption at 505nm.

The amount of H_2O_2 produced from test samples was calculated from a calibration curve of absorption at 505nm (λmax) against H_2O_2 concentration (Figure 3.15). Table 3.19 summarises the amount of H_2O_2 formed from the active metabolites of azo dyes and for their predicted end-products. Under the conditions described no H_2O_2 was detected for unreduced azo dyes and their aminonaphthalene products. Addition of reduced azo dyes or aminonaphthols to the test system produced small amounts of H_2O_2 as determined by an increase in absorption. Addition of increasing quantities of aminonaphthol produced increasing amounts of catalysed oxidation. The velocity of the formation of H_2O_2 depended upon the concentration of active metabolites which decreased with time. Figure 3.16a&b is a graphical representation of hydrogen peroxide concentration from reduced dyes and their predicted end-products.

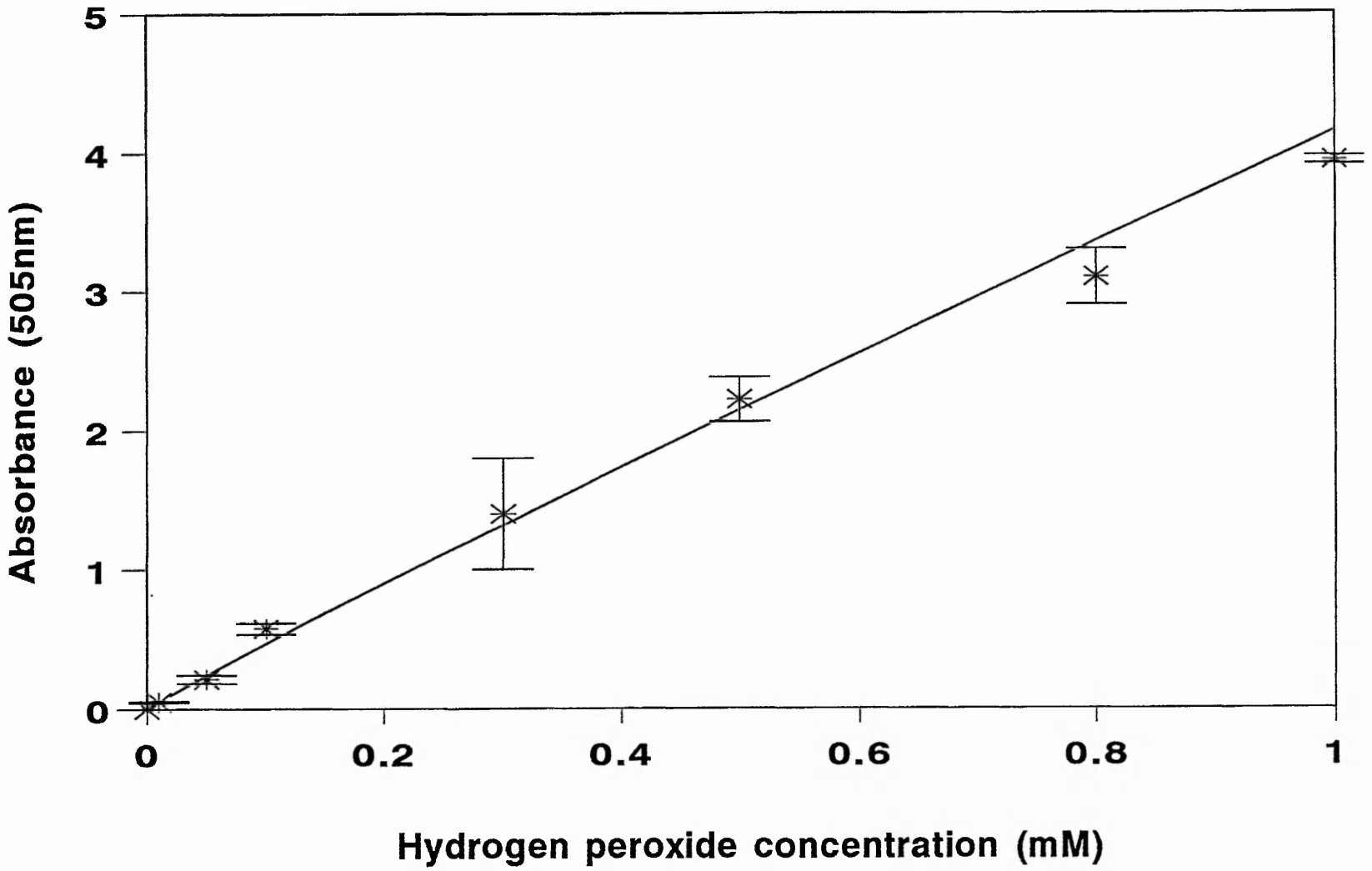


Figure 3.15 Hydrogen peroxide calibration curve

TABLE 3.19 Generation of hydrogen peroxide from azo dyes

Dye	Rate of hydrogen peroxide production/μmol compound/min		
	Azo reduction by		
	E. faecalis	*B. thetaiotaomicron*	Stannous Chloride
Amaranth	0.02 ± 0.004	0.03 ± 0.001	0.11 ± 0.04
Sunset yellow	0.06 ± 0.02	0.05 ± 0.007	0.13 ± 0.03
Carmoisine	0.01 ± 0.001	0.0	0.08 ± 0.03

TABLE 3.19 Generation of hydrogen peroxide from predicted end-products (Continued)

Predicted End-product	Rate of hydrogen peroxide production/μmol compound/min
1-Amino-2-naphthol	0.016 ± 0.01
1-Amino-2-naphthol-4-sulphonate	0.06 ± 0.004
1-Amino-2-naphthol-6-sulphonate	0.08 ± 0.007
1-Amino-2-naphthol-3,6-sulphonate	0.07 ± 0.01
1-Amino-naphthalene-4-sulphonate	0.0

Mean ± SD of 4 independent experiments

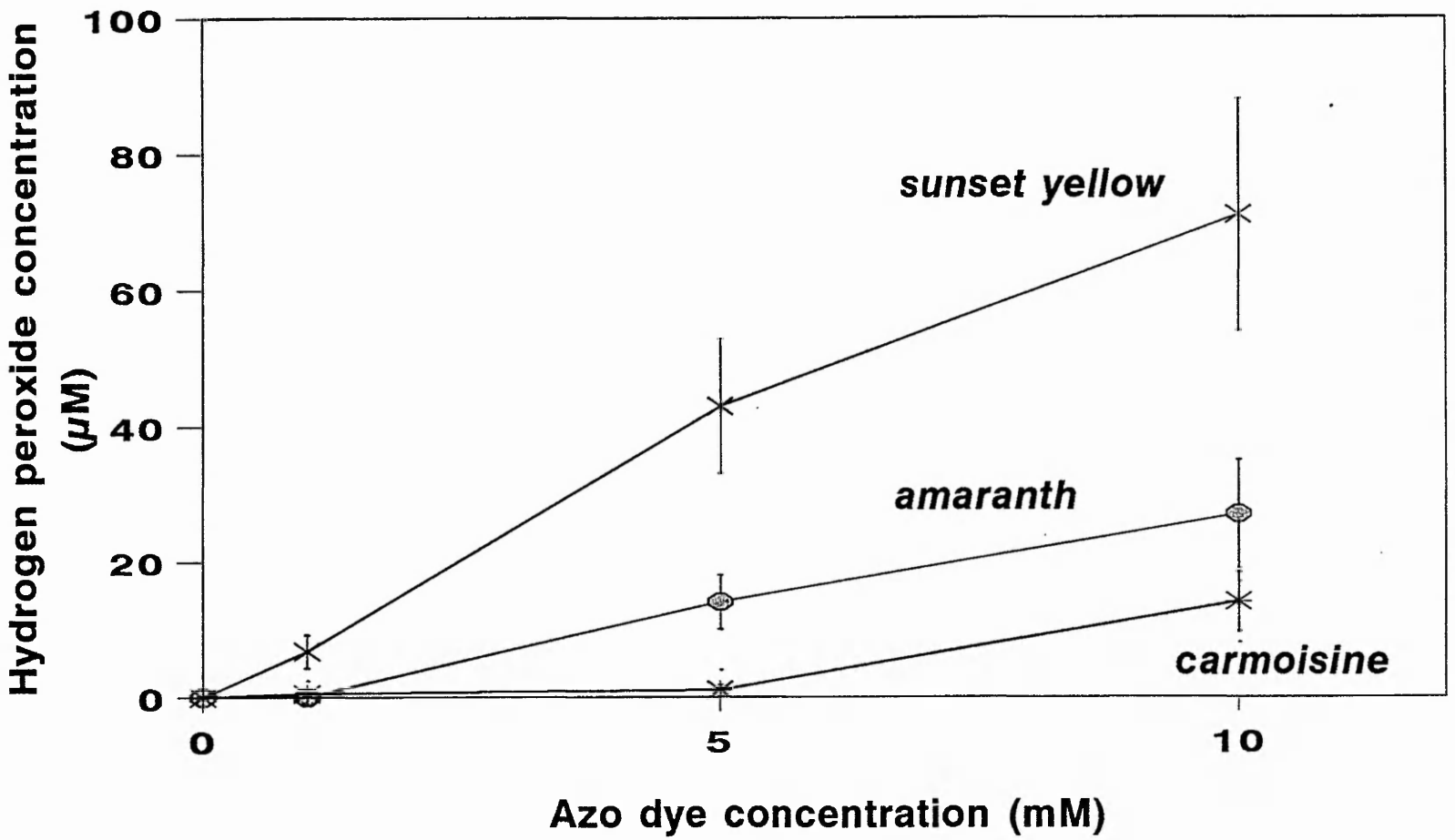


Figure 3.16a Hydrogen peroxide production from *E. faecalis* reduced azo dyes

Data are means +/- SD of 4 independent experiments

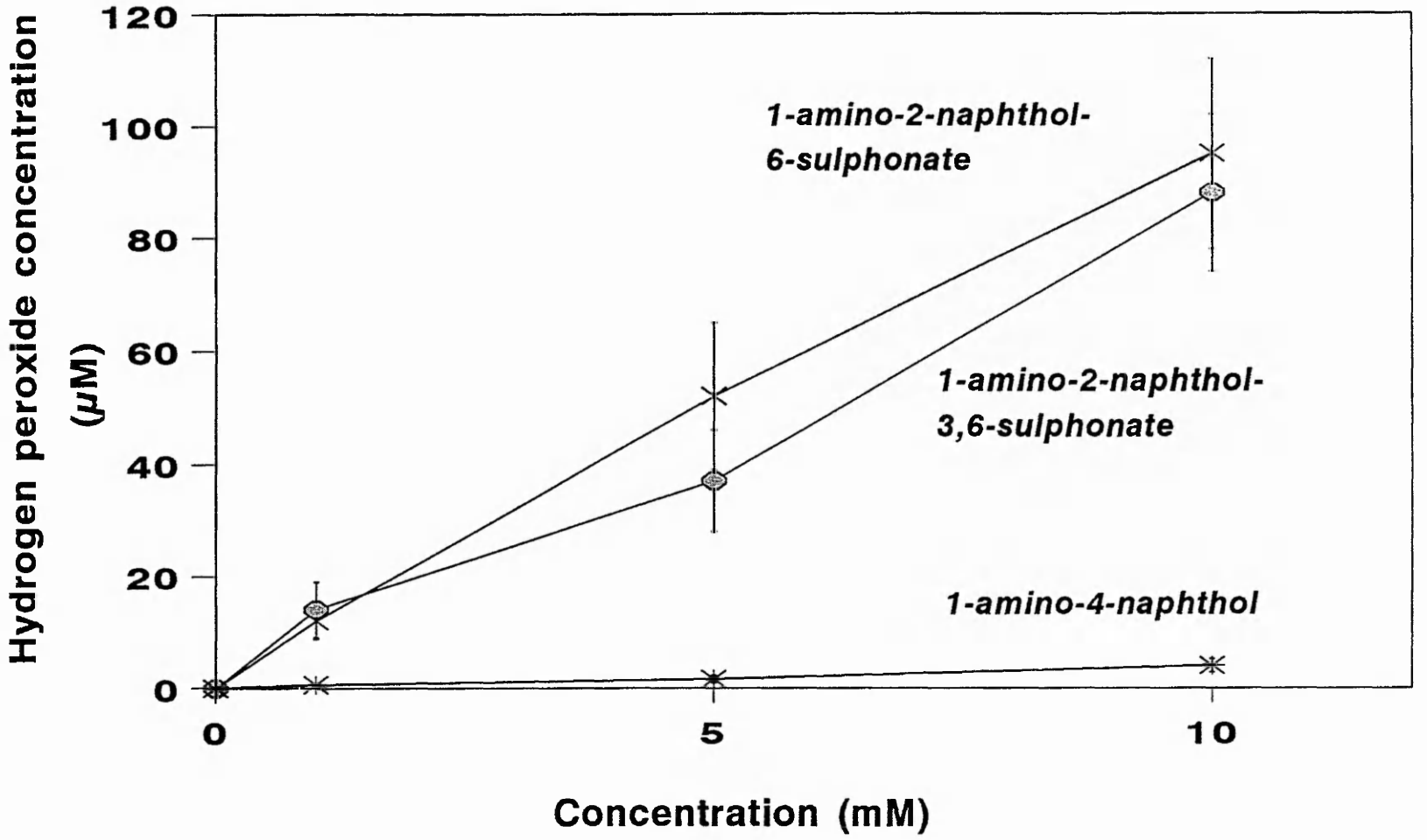


Figure 3.16b Hydrogen peroxide production from predicted end-products

Data are mean +/- SD of 4 independent experiments

3.9 DETECTION OF OXIDATIVE DNA DAMAGE

Free radicals produce a broad spectrum of DNA damage. The inherent problem associated with detecting low levels of oxidatively damaged bases in DNA has been resolved by the identification of 8-hydroxy-deoxyguanosine (8-OHdG). 8-OHdG can be quantitated to a high degree of sensitivity by electrochemical detection of enzymatic hydrolysates of DNA by reverse phase HPLC.

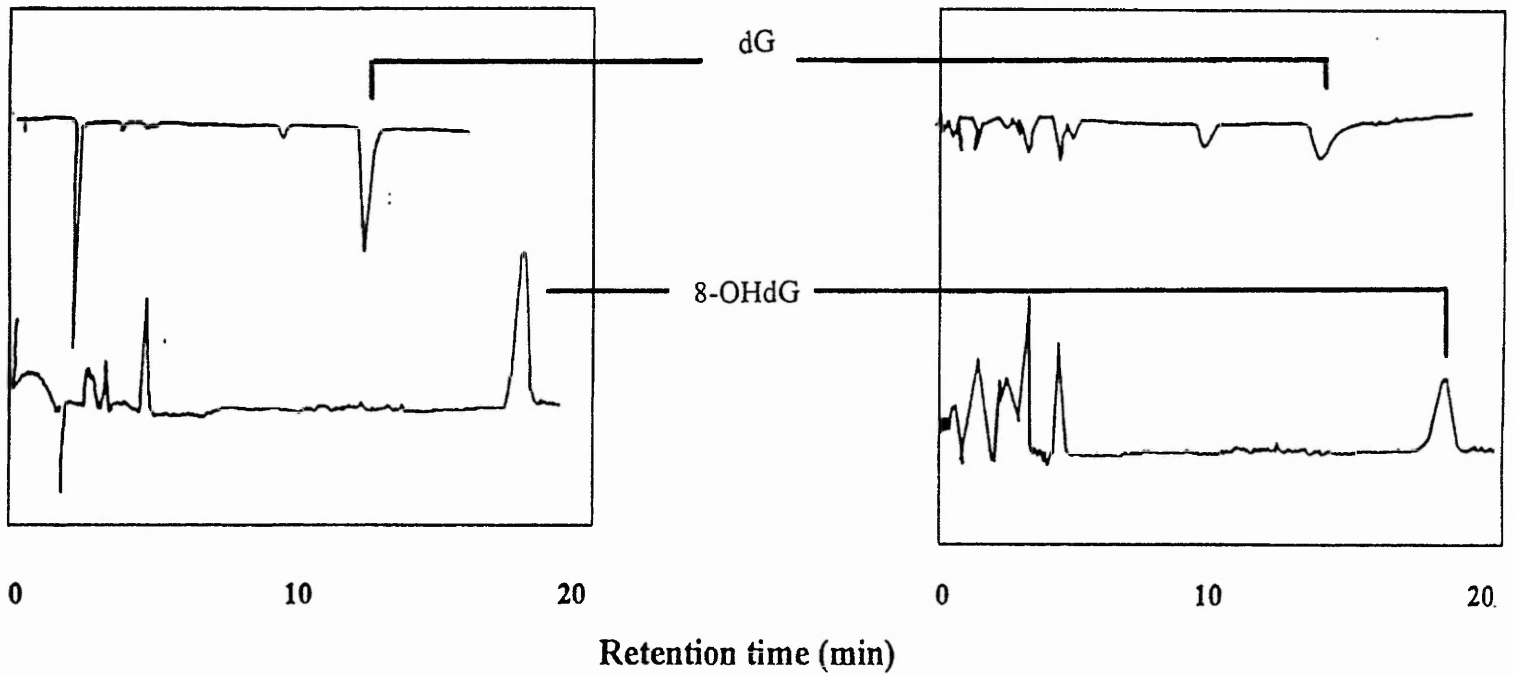
The principal aim of our studies was to determine whether there was a discernible difference between endogenous levels of 8-OHdG in DNA and levels of 8-OHdG formed in DNA subsequent to treatment with an oxygen radicals.

3.9.1 Effect of treating calf thymus DNA with test compound

Representative chromatographic profiles of 8-OHdG found in untreated and treated DNA are shown in Figure 3.17. Background levels of calf thymus DNA, not deliberately subjected to oxidative stress, were in the range of 0.02 - 0.085% molar ratio (8-OHdG/dG).

Hydrogen peroxide is thought to participate in a Fenton like reaction with transition metals, which are readily bound to DNA in trace quantities, resulting in the production of hydroxyl radicals close to the DNA. This proposed mechanism was examined by exposing DNA to hydrogen peroxide either in the presence of a hydroxyl radical scavenger or following pretreatment of DNA with metal chelators. The result indicated that trace quantities of transition metal ions can react with hydrogen peroxide to produce radical species. The yields of these products were dependent upon hydrogen peroxide concentration. The production of radical species was shown by increased levels of 8-OHdG. Figure 3.18 illustrates that the yield of 8-OHdG increased linearly with hydrogen peroxide concentration.

Incubation of DNA with amaranth caused no change in 8-OHdG level. When DNA was treated with *E. faecalis*-reduced amaranth there was an increase in levels of 8-OHdG with increasing sample concentration (Table 3.20).



Formation of 8-OHdG in calf thymus DNA treated with 1-amino-2-naphthol-3,6-disulphonate

ii) **Formation of 8-OHdG in *E.coli* DNA treated with 1-amino-2-naphthol-3,6-disulphonate**

Figure 3.17 HPLC profile of 8-hydroxydeoxyguaosine

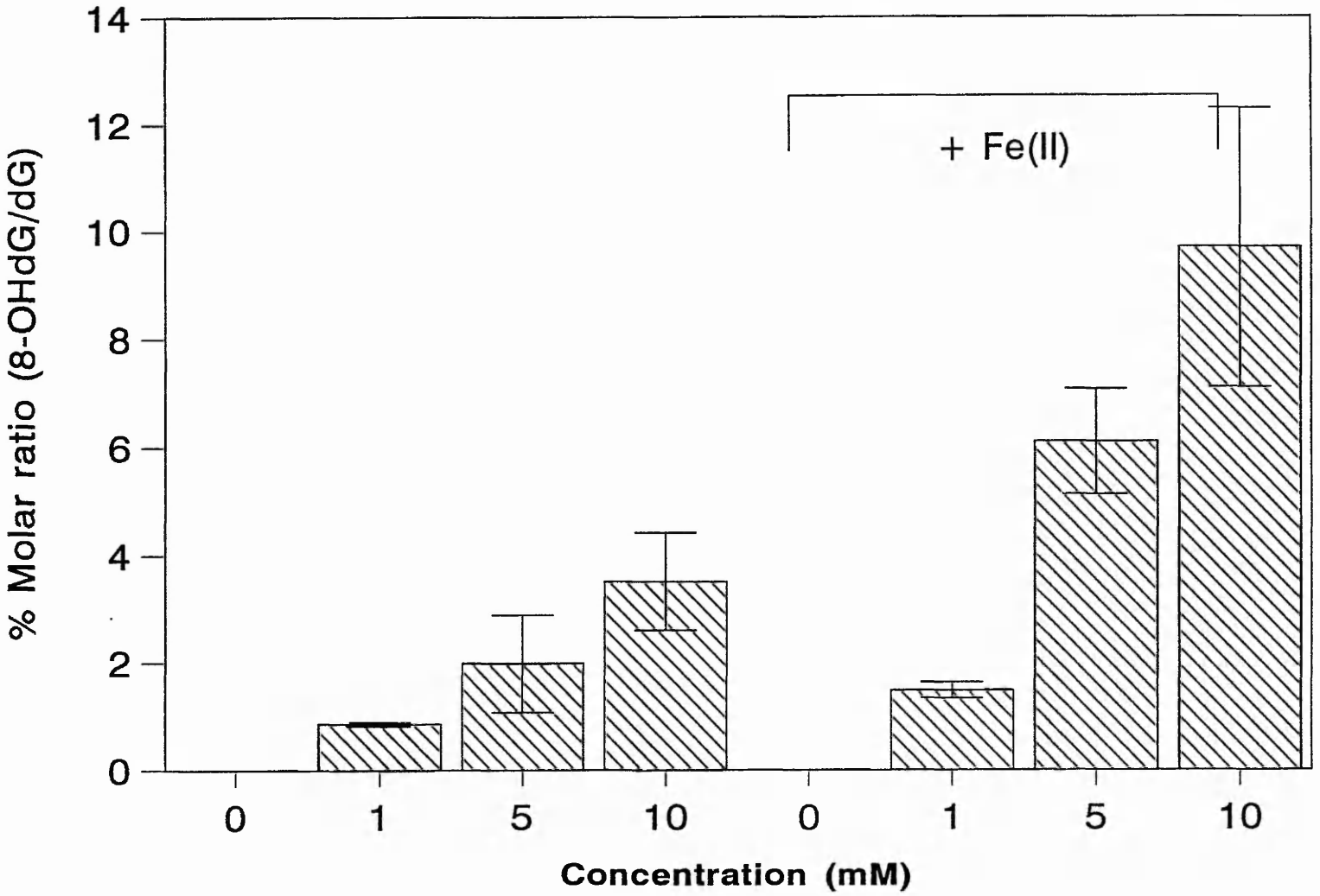


Figure 3.18 Formation of 8-OHdG in calf thymus DNA treated with hydrogen peroxide

Data are MEANS +/- SD of 4 independent experiments

TABLE 3.20 The detection of 8-OHdG in calf thymus DNA

Calf thymus DNA and hydrogen peroxide

Hydrogen peroxide concentration (mM)	% Molar Ratio (8-OHdG/dG)		
	No Fe^{2+} present	Fe^{2+} present	Catalase
0	0.002 ± 0.003	0.006 ± 0.001	0.002 ± 0.005
1.0	0.86 ± 0.04	1.48 ± 0.15	0.030 ± 0.01
5.0	1.98 ± 0.91	6.1 ± 0.97	0.102 ± 0.01
10.0	3.50 ± 0.91	9.70 ± 2.60	0.160 ± 0.024

Calf thymus DNA and amaranth (Fe present)

Amaranth concentration (mM)	% Molar Ratio (8-OHdG/dG)	
	Amaranth	*E. faecalis*-reduced Amaranth
0	0.0085 ± 0.002	0.0085 ± 0.002
0.1	0.0079 ± 0.005	0.034 ± 0.012
1.0	0.0073 ± 0.007	0.155 ± 0.005
5.0	0.0051 ± 0.012	0.823 ± 0.15
10.0	0.0040 ± 0.001	1.12 ± 0.14

Mean ± SD of 4 independent experiments

TABLE 3.20 The detection of 8-OHdG in calf thymus DNA (Continued)

Calf thymus DNA and 1-amino-2-naphthol-3,6 disulphonate

1-amino-2-naphthol-3,6 disulphonate concentration (mM)	% Molar Ratio (8-OHdG/dG)		
	No Fe^{2+} present	Fe^{2+} present	Catalase
0	0.0085 ± 0.002	0.009 ± 0.005	0.0041 ± 0.006
0.1	0.030 ± 0.005	0.050 ± 0.010	0.0092 ± 0.004
1.0	0.490 ± 0.020	1.05 ± 0.150	0.0037 ± 0.010
5.0	1.575 ± 0.080	3.38 ± 0.550	0.0110 ± 0.010
10.0	2.200 ± 0.100	6.23 ± 0.800	0.0170 ± 0.013

Mean ± SD of 4 independent experiments

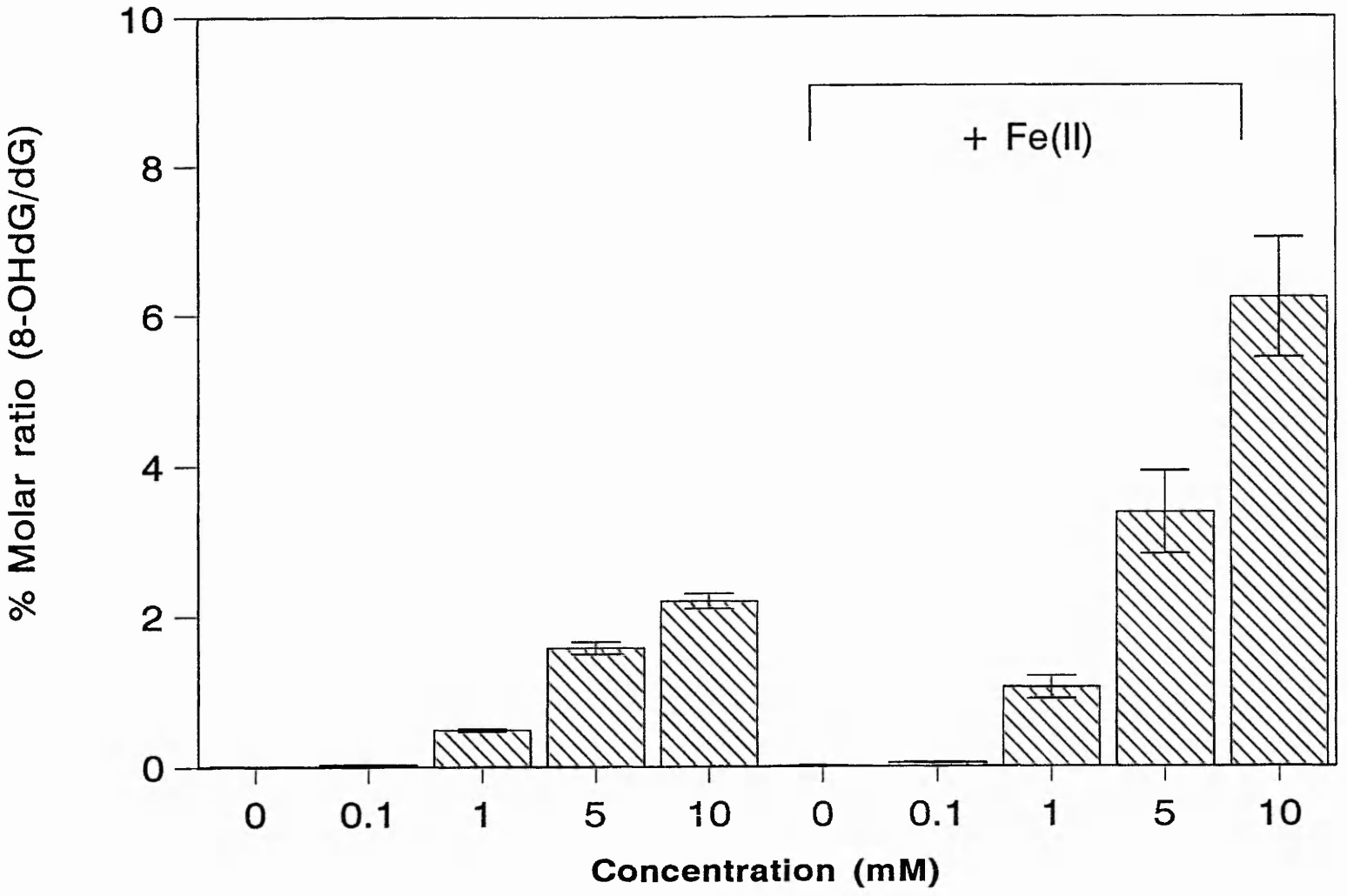


Figure 3.19 Formation of 8-OHdG in calf thymus DNA treated with 1-amino-2-naphthol-3,6-disulphonate

Data are MEANS +/- SD of 4 independent experiments

1-Amino-2-naphthol-3,6-disulphonate was significantly more affective than reduced amaranth at producing 8-OHdG in DNA (Figure 3.19) Although this product was not as effective as hydrogen peroxide in producing oxygen radical damage. The presence of Fe(II) enhanced the production of 8-OHdG.

3.9.2 Effect of scavenger on 8-OHdG production in calf thymus DNA

The effect of catalase, a hydrogen peroxide scavenger, on hydrogen peroxide-induced base damage was examined. When DNA was treated with hydrogen peroxide in the presence of catalase (final conc. 1mg/ml) there was over 70% reduction in 8-OHdG measured. Further increases in catalase concentration did not reduce the yields of these products.

The effect of catalase on 1-amino-2-naphthol-3,6-disulphonate induced base damage was also examined. Catalase inhibited the production of 8-OHdG. These results suggest that the mechanism behind base damage induced by 1-amino-2-naphthol-3,6-disulphonate is similar to that of hydrogen peroxide, that is through the Fenton reaction.

3.9.3 Effect of pretreating DNA with chelators

The influence of DNA bound iron ions on hydrogen peroxide-induced base damage was investigated by examining the effect of DNA pretreatment with metal ion chelators. In these experiments chelator-treated DNA solutions were dialysed to remove low molecular weight compounds, including the metal ion chelator complexes. Pretreatment of DNA with desferrioxamine inhibited formation of 8-OHdG in hydrogen peroxide treated DNA. Figure 3.20 and 3.21 illustrate these results. Levels of 8-OHdG were significantly lower after pretreatment of DNA with desferrioxamine prior to incubation with 1-amino-2-naphthol-3,6-disulphonate.

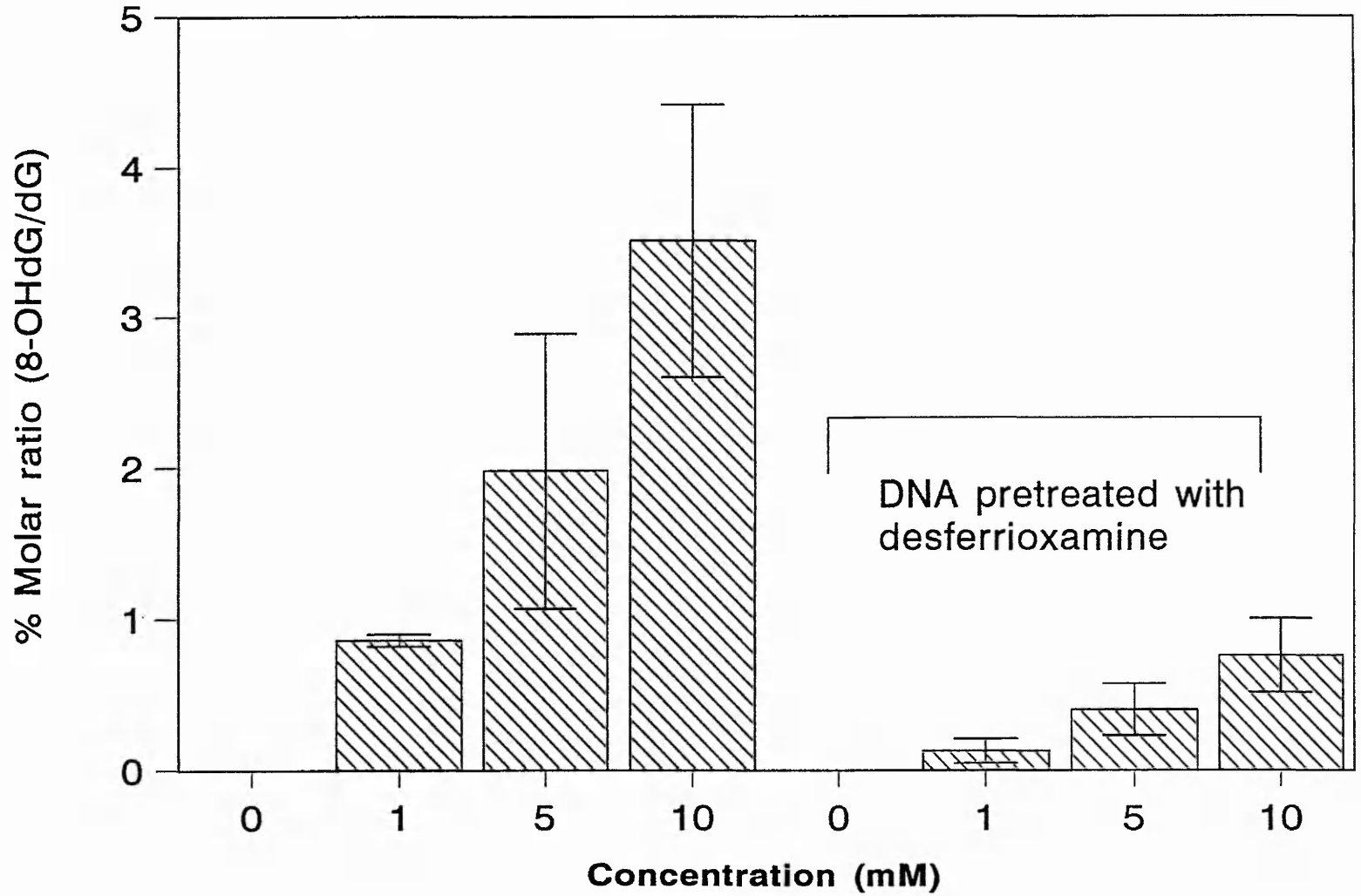


Figure 3.20 Formation of 8-OHdG in calf thymus DNA treated with hydrogen peroxide

Data are MEANS +/- SD of 4 independent experiments

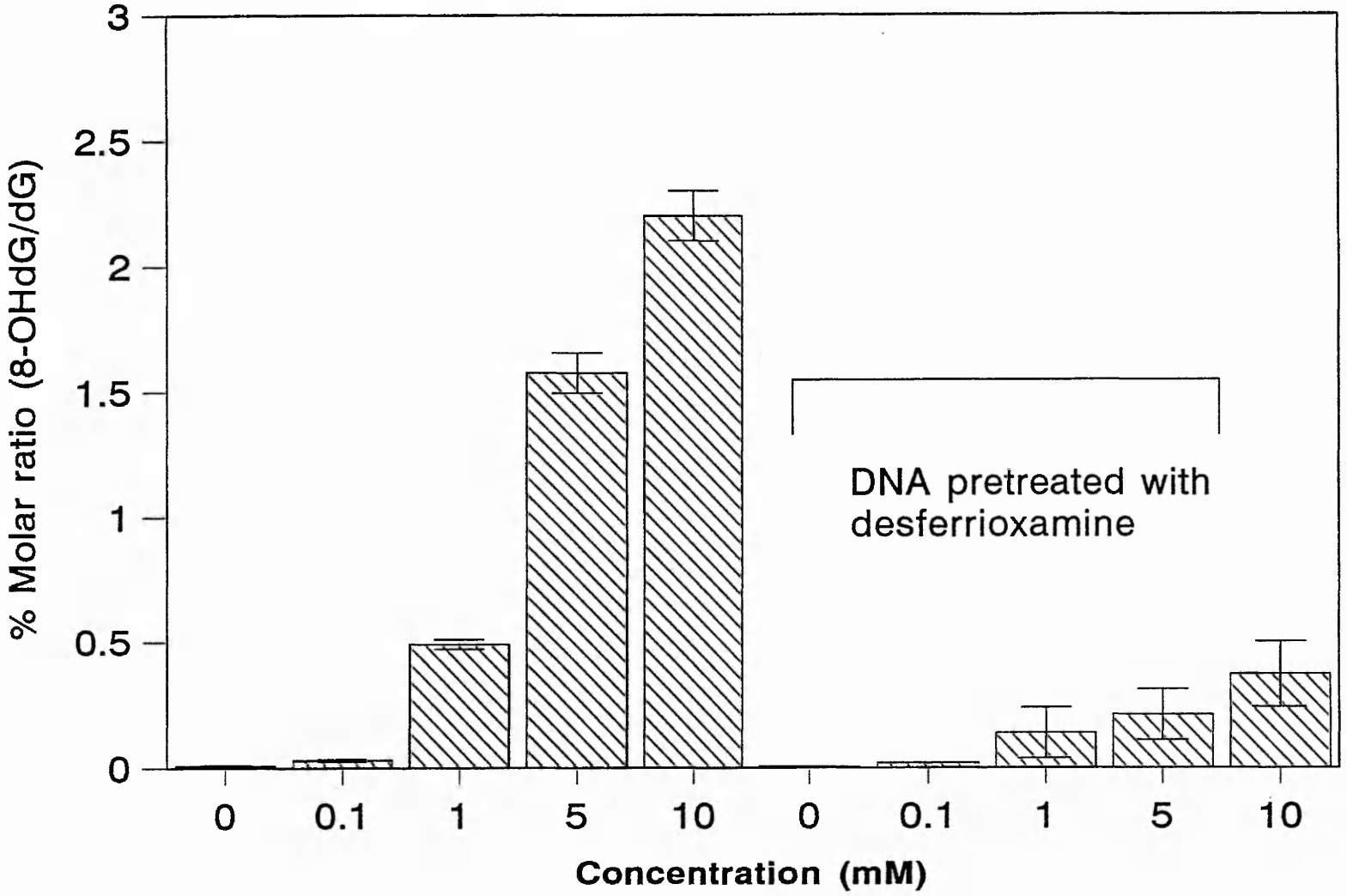


Figure 3.21 Formation of 8-OHdG in calf thymus DNA treated with 1-amino-2-naphthol-3,6-disulphonate

Data are MEANS +/- SD of 4 independent experiments

3.9.4 Effect of treating *E. coli* DNA with test compound

DNA was isolated from *E. coli* AB1157 and BH20 and the quantity of 8-OHdG determined. Hydrolysed and derivatised DNA samples were analysed by electrochemical HPLC. Only a moderate rise in levels of 8-OHdG were observed in *E. coli* AB1157 as the hydrogen peroxide concentration increased. This may indicate that the 8-OHdG lesions are being excised very effectively. HPLC-EC analysis of released 8-OHdG from *E.coli* DNA treated by hydrogen peroxide, reduced amaranth and 1-amino-2-naphthol,3,6-disulphonate are shown in Tables 3.21 and 3.22. Confirmation of the identity of the released nucleoside was achieved by comparison of HPLC-EC elution profiles of its authentic standard and electrochemical response (hydrodynamic voltammogram). An enzymic hydrolysate of DNA following HPLC produced a dG peak absorbing at 260nm. 8-OHdG produced a detectable signal at 0.6V of electrochemical detection.

There was a discernible difference between levels of 8-OHdG in *E. coli* AB1157 and BH20. Endogenous levels of 8-OHdG in DNA of bacteria were also measured. The results gave an average of 0.013, 0.02 %molar ratio (8-OHdG/dG) for *E. coli* AB1157 and BH20 respectively (Table 3.21). The lower levels of 8-OHdG present in *E. coli* AB1157 DNA indicate the existence of repair activity. This suggests that the 8-OHdG residues in DNA are biologically undesirable to the cell. DNA repair is unlikely to remove all the lesions due to continuous sustained oxidative stress. Thus endogenous levels of 8-OHdG may reflect damage persisting in the DNA of an organism.

The results suggest that in normal conditions cellular DNA is damaged with formation of 8-OHdG by oxygen radicals that are generated by normal cellular metabolism. For the parent strain, *E. coli* AB1157, only moderate increases in the level of 8-OHdG were observed with increasing hydrogen peroxide. This is due to repair of 8-OHdG lesions by the fapy enzyme. As shown in Table 3.21, levels of 8-OHdG increased with increasing hydrogen peroxide concentration. The ability of hydrogen peroxide to produce 8-OHdG damage in *E. coli* DNA was confirmed by the protective effect of catalase.

TABLE 3.21 The detection of 8-OHdG in *E. coli* AB1157 & BH20 DNA

Hydrogen peroxide Concentration (mM)	%MOLAR RATIO (x10 $^{-3}$) (8-OHdG/dG)			
	E. coli AB1157		*E. coli* BH20	
	- catalase	+ catalase	- catalase	+ catalase
0	0.013 ± 8.0	0.012 ± 4.0	0.02 ± 4.0	0.017 ± 4.8
0.1	0.017 ± 4.0	0.009 ± 7.0	0.025 ± 2.0	0.019 ± 7.3
1.0	0.009 ± 2.0	0.015 ±6.0	0.029 ± 7.0	0.015 ± 52.5
5.0	0.012 ± 6.0	0.013 ±11.0	0.041 ± 5.0	0.016 ± 7.0
10.0	0.016 ± 11.0	0.012 ± 19.0	0.078 ± 9.0	0.017 ± 4.9

SD Standard Deviation
Represents mean values and standard deviations for four independent analyses

TABLE 3.22 The detection of 8-OHdG in *E. coli* DNA after preincubation with reduced dyes and their predicted end-products

Concentration (mM)	% Molar Ratio ($x10^{-3}$) (8-OHdG/dG)			
	Amaranth	*E. faecalis* reduced amaranth	1-amino-2-naphthol-3,6 disulphonate	1-amino-2-naphthol-3,6 disulphonate + catalase
***ESCHERICHIA COLI* AB1157 DNA (Fe present)**				
0	0.013 ± 0.0075	0.013 ± 0.007	0.013 ± 0.007	0.013 ± 0.007
0.1	0.014 ± 0.005	0.015 ± 0.006	0.025 ± 0.005	0.006 ± 0.001
1.0	0.013 ± 0.007	0.020 ± 0.003	0.045 ± 0.005	0.011 ± 0.002
5.0	0.021 ± 0.010	0.029 ± 0.004	0.051 ± 0.008	0.019 ± 0.005
10.0	0.009 ± 0.006	0.032 ± 0.001	0.060 ± 0.000	0.02 ± 0.008
***ESCHERICHIA COLI* BH20 DNA (Fe present)**				
0	0.019 ± 0.003	0.019 ± 0.003	0.020 ± 0.004	0.015 ± 0.002
0.1	0.021 ± 0.005	0.030 ± 0.002	0.075 ± 0.015	0.04 ± 0.004
1.0	0.017 ±0.010	0.040 ± 0.010	0.160 ± 0.060	0.075 ± 0.01
5.0	0.013 ± 0.004	0.095 ± 0.019	0.470 ± 0.015	0.23 ± 0.050
10.0	0.025 ± 0.002	0.134 ± 0.040	0.570 ± 0.000	0.34 ± 0.030

SD Standard Deviation
Represents mean values and standard deviations for four independent analyses

Results obtained for *E. coli* DNA treated with amaranth and its azo reduction products, were comparable with those obtained in calf thymus DNA (Table 3.22). The levels of 8-OHdG were not as high as those obtained in calf thymus DNA. This may be due to cells possessing mechanisms, including sufficient DNA repair capacity, to minimise the affects of normal levels of oxidative damage.

3.9.5 Effect of scavenger on 8-OHdG production in *E. coli* DNA

When *E. coli* was treated with test compound in the presence of extracellular catalase, concentration of 8-OHdG dropped significantly (Figure 3.22). This reiterates results obtained for calf thymus DNA.

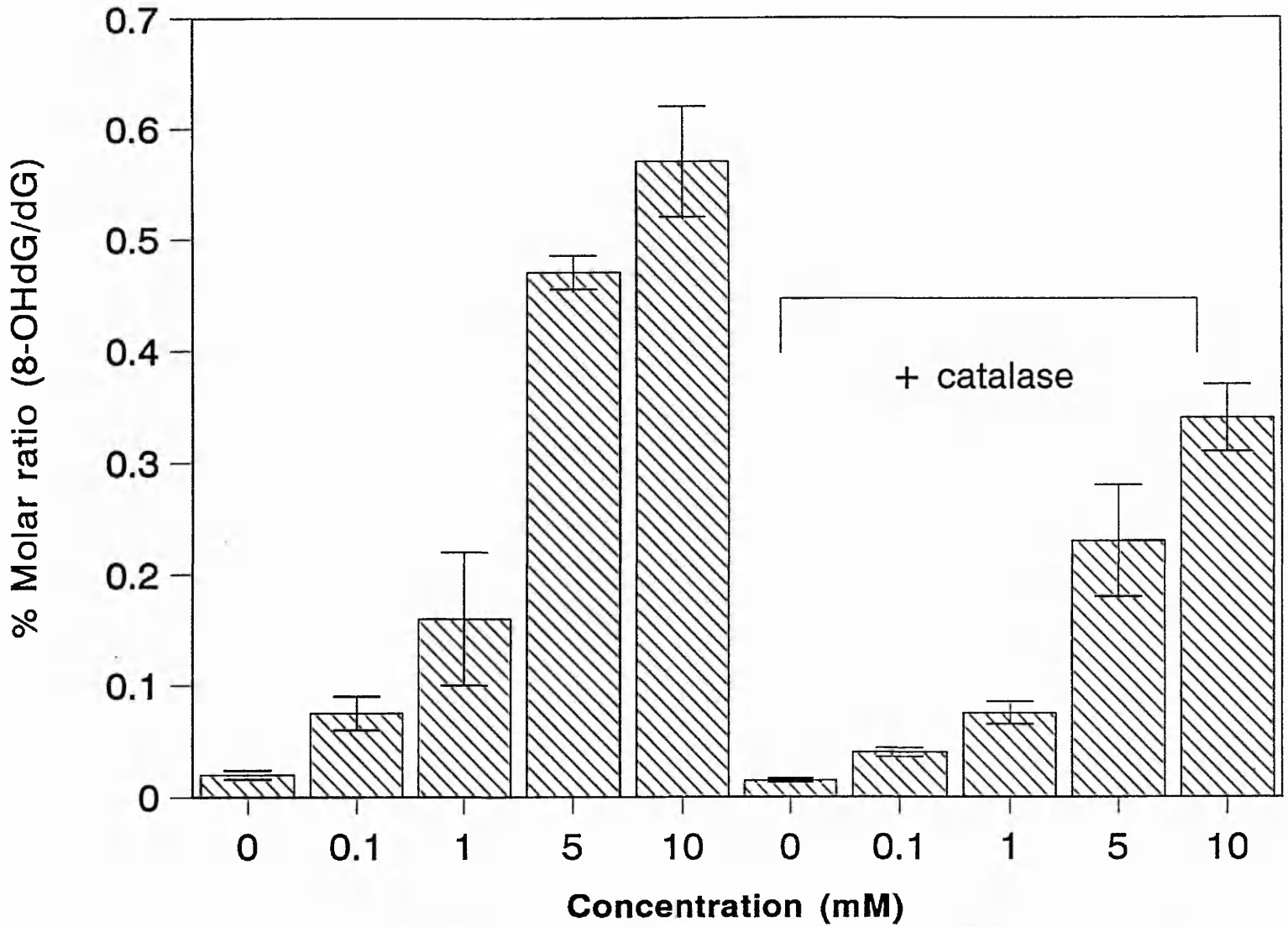


Figure 3.22 Formation of 8-OHdG in *E. coli* DNA treated with 1-amino-2-naphthol-3,6-disulphonate

Data are MEANS +/- SD of 4 independent experiments

CHAPTER 4

DISCUSSION

DISCUSSION

4.1 AZO REDUCTION

A major advance in the understanding of the metabolism of water soluble azo dyes resulted from the work of Radomski and Millinger (Walker, 1970), who demonstrated the great importance of modifications taking place in the gut of experimental animals before absorption. Using antibiotics, these workers were able to show that the observed reductive fission of the azo group in the gut was probably due to the action of the gut microflora. Childs *et al.* (1967) studied the azo reducing systems of the rat gut. The intestinal contents, tissue and bacterial isolates showed azo reducing activity, whereas the stomach and duodenal contents did not. It was therefore concluded that the observed activity was microbial in origin.

Chung *et al.* (1978) demonstrated that seven azo dyes (amaranth, orange II, ponceau SX, allura red, sunset yellow, tartrazine and methyl orange) were reduced by a variety of intestinal bacteria including *Fusobacterium* sp.2, *B. thetaiotaomicron, Peptostreptococcus productus* and *Citrobacter* sp.. Others, such as *Peptostreptococcus productis* II and *Bifidobacterium infantis* reduced only a few azo dyes. The results from this study indicate that the reduction of azo compounds can be accomplished by the major anaerobes rather than by a few facultative micro-organisms in the gastrointestinal tract. Additionally, azo reduction appears to have a wide species and substrate specificity.

Rates of azo reduction vary with the dye substrate. Chung *et al.* (1978) determined the % reduction (within 150 minutes) of amaranth by *B. thetaiotaomicron* to be 53%, while for *Citrobacter* sp. and *Fusobacterium* sp. 2, % reduction was 66% and 100 % respectively. For Sunset Yellow, % reduction was 60%, 56% and 100% for *B. thetaiotaomicron, Citrobacter* sp. and *Fusobacterium* sp. 2.

Since then many studies have been conducted on the reduction of azo compounds by anaerobic intestinal bacteria. Cerniglia and co-workers, (1982) using monocultures of *Bacteroides, Bifidobacterium, Citrobacter, Clostridium, Lactobacillus, Peptococcus, Peptostreptococcus* and

Escherichia species, demonstrated the ability of these intestinal micro-organisms to reduce the azo dyes, direct red 2 and direct blue 15, to their respective free amines. Chung (1983) provided numerous examples of microbial mediated metabolism of several azo dyes and their constitutive aromatic amines (Figure 4.1).

Our studies showed inhibition by glucose on azoreductase activity of *B. thetaiotaomicron.* Conversely the presence of glucose enhanced azoreductase activity of *E. faecalis* (Table 3.1). Roxon *et al.* (1966) reported that glucose stimulated the reduction of tartrazine by *P. vulgaris.* In contrast, the effect of glucose on tartrazine reduction by *B. thetaiotaomicron* was inhibitory at concentrations as low as 1μM for Chung *et al.* (1978). The significance of this marked difference is not clear at present. Chung and Stevens (1992) proposed that when ingested fibre reaches the colon, it is partially digested and releases soluble sugars like glucose. Glucose may inhibit azoreductase activity, thus decreasing the new production of aromatic amines which are potential carcinogens in the gastrointestinal tract. This may partially account for the fact that certain colonic disorders such as cancer of the colon or rectum may be due to a deficiency of dietary fibre in the diet. However this does not account for the stimulation of azoreductase activity in *Proteus* sp. and *E. faecalis* in the presence of glucose. Goldin and Gorbach (1976) also showed that the addition of fibre to the diet of omnivores for 30 days had no affect on the azoreductase activity of their faecal microflora.

Reduction of amaranth, carmoisine and sunset yellow by a washed cell suspension of *E. faecalis* was stimulated by exogenous FMN or RF (Table 3.1). Deleting FMN from the incubation mixture resulted in a much lower level of reducing power. In the case of *B. thetaiotaomicron,* azo reduction did not occur in the absence of added flavins. Since this factor was critical from the quantitative assay we determined the effect of various FMN concentrations on the reduction of the azo dye. Diminishing the amount of FMN in the assay caused a gradual but steady decrease in azoreductase activity of the cell (Figure 3.1). The inclusion of this cofactor was essential for maximum activity. RF and FAD were inferior to FMN as cofactors for azo dye reduction.

These results support those of Chung *et al.* (1978), who found that the addition of flavin mononucleotide to the reaction mixture caused a marked linear enhancement of azo reduction of

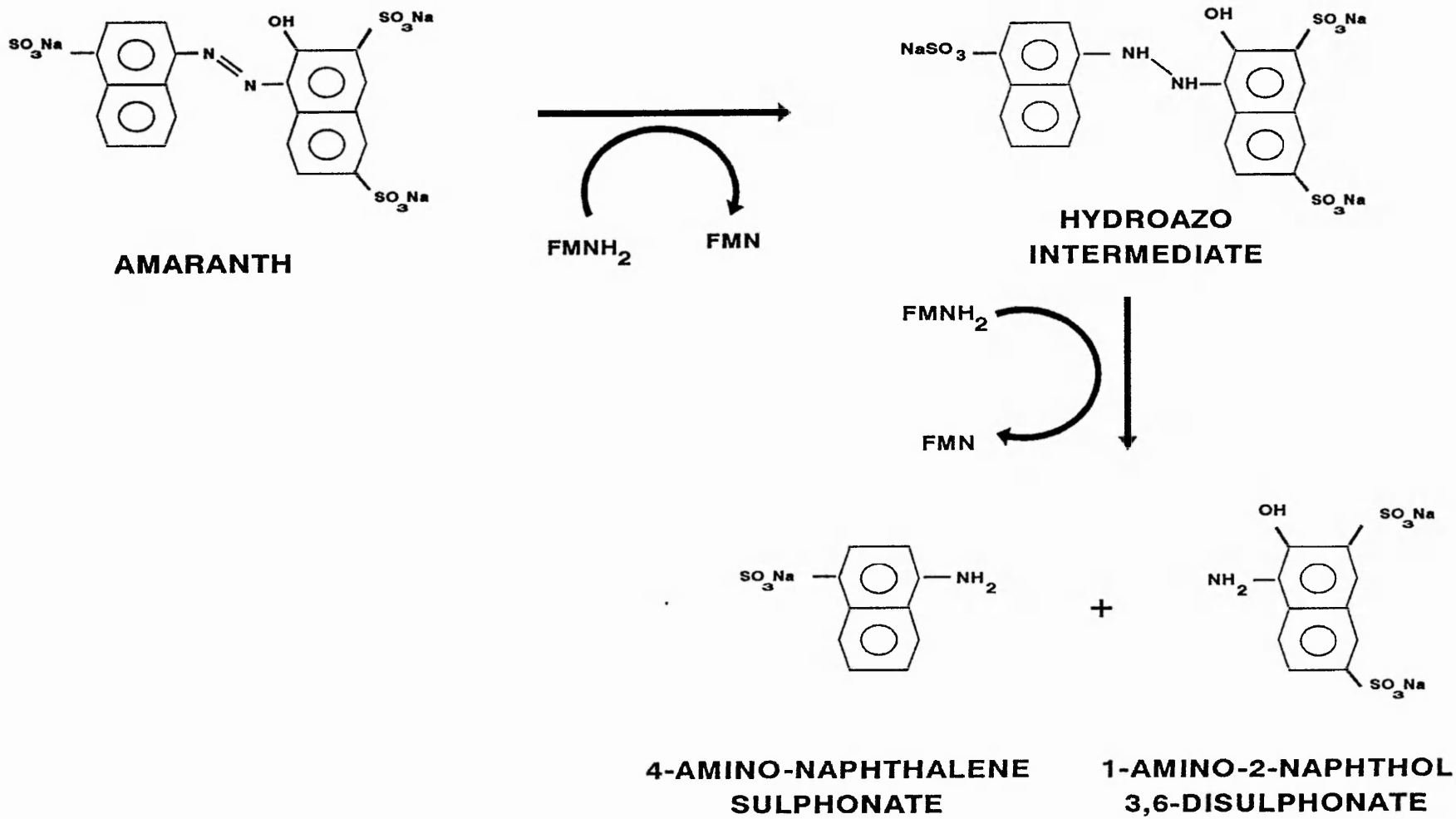


FIGURE 4.1 Proposed mechanism for the bacterial reduction of amaranth

tartrazine by *B. thetaiotaomicron.* Chung also found all the following electron carriers stimulated azo reduction: methyl viologen, benzyl viologen, phenosafranin, neutral red, crystal violet, flavin adenine dinucleotide, menadione and janus green B. They concluded that extracellular acceptors stimulate azo dye reduction. The reduction occurs anaerobically, and thus the gastrointestinal tract, particularly the colon, which is the most anaerobic environment in the body, is probably the primary site for the reduction of azo dyes.

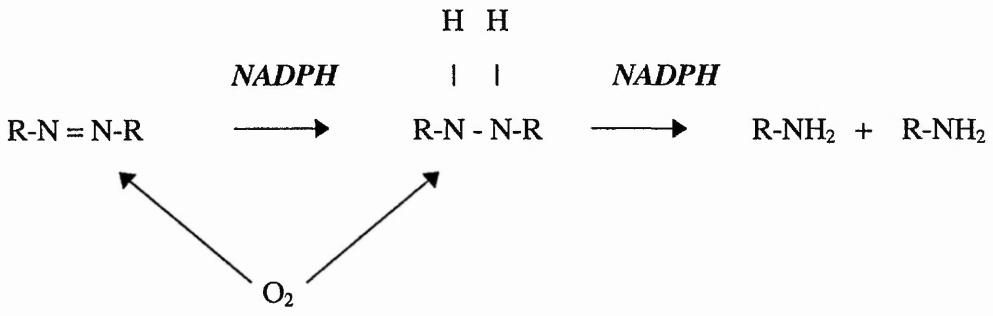
Previous studies on the mechanism of reduction of azo compounds by common components of the intestinal microflora such as *P. vulgaris* and *E. faecalis* also have indicated that reduction occurs via enzymic generated reduced flavins, but that the final step in the transfer of electrons occurs non-enzymically (Walker and Ryan, 1971). It is possible that flavin acts as a shuttle between the dye and bacterial enzyme. A double two electron transfer via the hydrazo intermediate would be more likely in azo reduction.

$$FMNH_2 + R\text{-}N{=}N\text{-}R' \rightarrow FMN + R\text{-}NH\text{-}NH\text{-}R'$$

$$FMNH_2 + R\text{-}NH\text{-}NH\text{-}R' \rightarrow FMN + R\text{-}NH_2 + R'NH_2$$

In view of their charged nature, it is unlikely that FAD or FMN could readily shuttle across bacterial cell walls and membranes under physiological conditions. However RF may fit such a role. This concept is helpful in rationalising reduction of many highly charged sulphonated dyes by intact bacterial cells since it is unlikely that such dyes could pass through the cell wall and plasma membrane. An electron shuttle could effect the reduction with the dyes remaining extracellular. It may also explain the oxygen sensitivity of these reductions if one assumes that reduced flavin has a greater affinity for oxygen than for dye. Brief exposure of *E. faecalis* cell-free extracts to oxygen resulted in complete loss of azoreductase activity. The sensitivity of azo reduction to oxygen may be understood as a competition of oxidants for reduced electron carriers in the respiration chain by oxygen and azo compounds. In the absence of oxygen, an azo compound will act as sole oxidant, and its reduction rate will be governed by the rate of formation of the electron donor, i.e. a reduced flavin nucleotide. In the presence of oxygen, however, the reduced flavin nucleotides will be competitively oxidised and the rate of azo reduction concomitantly slowed.

Inhibition of azo reduction by oxygen may also be caused by the reoxidation of hydrazo intermediates, which presumably are formed in the reduction of azo compounds. The oxidation of the hydrazo intermediate may also cause a depletion of NADPH and thus may result in poor yield of the primary amine according to the following scheme:



Although oxygen is often an inhibitor of azo reduction, there are dyes which can be degraded aerobically by some micro-organisms. *p*-Aminoazobenzene was reduced by *B. subtilis* under experimental conditions that were aerobic or at least microaerophilic (Horitsu *et al.*, 1977). Liu and Yang (1989) demonstrated that oxygen inhibited azo reduction by a purified azoreductase and not by whole cell suspensions of *Pseudomonas* sp. S-42. Thus although anaerobiosis does not appear crucial for azo reduction, it is generally a favourable condition for this activity.

Studies on the mechanism of azo reduction by *E. faecalis* showed there was a lag period before reduction commenced. This delay in reduction occurred despite rigorous precautions to exclude oxygen which inhibits azo reduction. It is likely this lag period was due to enzymatic regeneration of a reduced cofactor before dye reduction commenced. There was an increase in microbial azoreductase activity associated with increased flavin concentration (Figure 3.1). Continuous estimation of dye reduction rate also showed that the rate decreased when the dye concentration became low.

Sodium azide, which complexes with respiratory cytochromes, had little effect on azoreductase activity of *E. faecalis* confirming that cytochromes were probably not involved in this azo

reduction. HQNO, a flavin analogue and competitive inhibitor of flavoprotein enzymes inhibited azo reduction. Therefore the protein responsible for flavin reduction and thus azo reduction was probably a flavoprotein. It has been demonstrated that there are no cytochromes present in cell free extracts of *E. faecalis* and that the transfer of electrons from reduced pyridine nucleotides to electron acceptors was mediated by a flavoprotein (Gingell and Walker, 1971). Sodium azide, which complexes with respiratory cytochromes, had little effect on azo reduction confirming that cytochromes are probably not involved in this azo reduction. HQNO, a flavin analogue and competitive inhibitor of flavoprotein enzymes inhibited azo reduction.

The rate of reduction was also affected by molecular parameters influencing the ease with which electrons are accepted by the azo group. In this study, fourteen azo compounds were used as substrates to study the influence of aromatic substitution patterns on azo dye degradation by *E. faecalis* (Table 3.4). Decolouration did not occur when the hydroxy group was in the para position relative to the azo linkage and there were no electron releasing substituents (azobenzene and *p*-phenylazo phenol). Orange I which has a hydroxy group para to the azo linkage and has an electron releasing substituent (sulphonate group) was reduced. In all cases, the azo dyes containing a sulphonate group peri (position 8) to the azo linkage were not reduced. Its close proximity to the azo group suggests the possibility of steric hindrance of the dye to the enzyme and protection of the azo linkage to reduction.

The results also showed that for azo dyes with the hydroxyl group ortho to the azo bond, decolouration was considerably enhanced by the substitution of electron withdrawing groups (sulphonate groups) on the phenyl or naphthol ring. Substitution of a sulphonate group in the para position of the phenyl of naphthalene ring did not significantly affect the rate of reduction, but the substitution of electron withdrawing groups in position 3 or 6 of the naphthol ring resulted in a marked increase in reduction rate in both phenyl-naphthol and naphthalene-naphthol dyes. The presence of two naphthol rings result in very little reduction. These results paralleled findings for disazo dyes. The presence of sulphonate groups enhanced reduction (Table 3.4).

From the results of each azo dye structure, factors other than lipid solubility must also affect the rate of azoreductase activity. Enzymatic reduction of azo dyes may either be a one or two electron

transfer. Therefore the rate of reduction should be influenced by the electron density at the azo bond. Structural variations which can modify this electron density include the nature of the substituents around the ring systems and the potential for intramolecular hydrogen bonding of the substituents with the azo bond. For example, electron withdrawing groups will decrease the electron density, thereby enhancing azo bond reduction. Moreover, the hydroxy or amino groups in close proximity to the azo can enhance reduction by hydrogen bonding with the azo bond. Furthermore, the nature of the aromatic systems around the azo bond should affect the rate of azo reduction. Thus, naphthalene provides greater steric hindrance, which should decrease the azoreductase activity in comparison with a phenyl ring.

The results of this investigation demonstrated that sudan I had the slowest rate of azo dye reduction probably due to both the lack of electron withdrawing groups on the aromatic ring system and its high lipid solubility. Higher rates of reduction were observed for those dyes containing electron withdrawing substituents such as a sulphonate group in orange G. Amaranth had a high rate of azo reduction due to the presence of more electron withdrawing groups (sulphonates in the ring system). Apparently the larger the number of electron withdrawing groups the more rapid the rate of reduction by the micro-organisms. Amaranth had a lower rate of reduction than sunset yellow which was not unexpected due to the steric hindrance from the naphthalene ring of amaranth as opposed to the phenyl ring of sunset yellow.

Zbaida and colleagues (1994) also reported that electron donating substituents such as -OH, - NH_2, - $NHCH_3$ and - $N(CH_3)_2$ were prerequisites for reduction of azo benzene derivatives by microsomal cytochrome P-450. Azo compounds lacking electron donating substituents did not exhibit typical substrate cytochrome P-450 binding spectra or undergo microsomal reduction. Structure activity studies with related azo dyes by this group indicated that a polar electron donating substituent at the para position was essential for binding to cytochrome P450 and enzymic reduction. Thus compounds such as azobenzene do not exhibit typical substrate binding spectra in the presence of microsomes and were not reduced. The combination of both electron donating and withdrawing substituents in the sensitive substrates facilitates electron delocalisation throughout the molecule from the unshared pair of electrons of the electron donating group (i.e. OH group) towards the electron withdrawing group (i.e. sulphonate group). This is most pronounced when the

groups are ortho and para to the azo linkage. Electron delocalisation also stabilised both one and two electron reduced metabolites.

Larsen *et al.* (1976) also tried to demonstrate a relationship using four water soluble azo dyes differing in the number of sulphonate groups, and suggested that the reduction rate of an azo dye by rat-gut flora depended upon the number of sulphonate groups rather than on their positions in the dye molecule. They assumed that bacterial reduction would occur more readily in azo dye molecules in which the electron density at the azo linkage was lowered by substitution with a sulphonate group especially at the para or ortho position. However microbial reduction rates were actually lower in the azo dyes with more sulphonate groups. Larsen and his co-workers suggested that this discrepancy was probably due to the different abilities of these dyes to penetrate the bacterial cell wall. Their suggestion about the structural influence on the microbial reduction of sulphonated azo dyes, however does not fit in with results obtained by Watabe *et al.*, (1980). They found that amaranth with three sulphonate groups was reduced more readily than the disulphonate dye sunset yellow, and even more readily than new coccine, which is trisulphonated..

Larsen's results parallel the findings of Zimmermann *et al.* (1982), who reported on the properties of purified orange II azoreductase, an enzyme initiating azo dye degradation by *Pseudomonas* sp. strain KF46. They showed the specificity of the orange II azoreductase with regard to the position of the hydroxy group on the naphthol ring of the tested substrates. Orange dyes carrying a hydroxy group in other than the ortho position relative to the azo bond were not recognised by the purified orange II azoreductase. Moreover, they reported on an orange I azoreductase that would recognise only substrates having the hydroxy group in the para position. The also showed that many substituents on the azo dyes were or were not tolerated by orange II azoreductase and that electron withdrawing groups on the phenyl ring accelerated the enzyme action. Pasti-Grigsby *et al.* (1992), hypothesised that the azoreductase produced by *Streptomyces* sp. was similar to orange I azoreductase, since it was oxygen insensitive and specific for a hydroxy group in the para position relative to the azo bond.

From my results the predominant factor determining the reduction rate was electron density in the region of the azo group (Table 3.4). Stabilisation by hydrogen bonding was also important. The molecular parameters influencing the ease of azo reduction appeared to include:

i) The hydroxyl group ortho or para to the azo linkage was a prerequisite for reduction.

ii) Charged groups in proximity to the azo linkage hindered the reaction.

iii) The presence of electron withdrawing groups accelerated azo reduction.

iv) A second polar substituent on the dye molecule lowered its affinity for the enzyme.

v) Naphthalene-naphthol dyes were reduced more slowly than phenyl-naphthol dyes.

Thus bacterial reduction occurs more readily in azo dyes in which the electron density is lowered by substitution with a sulphonate group. Rate of reduction will depend on the position this group has within the dye molecule.

In 1980, Wuhrmann showed that sulphonic acid substitution of the azo dye structure blocked effective permeation. Treatment of *B. cereus* by toluene removed the block to dye permeation and resulted in the significantly increased passage of sulphonated and carboxylated azo dyes from external medium into the cell with a concomitant increase in the reduction rate of the dye. The rate of permeation of the dyes through the cell membrane appeared to be the limiting step in the microbial reduction of azo dyes. Dyes adsorbed by bacterial cell walls were in most cases reduced at slow rates and did not influence the simultaneous reduction of non-adsorbable dyes in the medium. Wuhrmann *et al.* (1980) also showed that all azo dyes not measurably reduced by whole cells of *B. cereus* were reduced by cell-free extracts. These observations suggested that membrane transport of the dye might be an important factor in the rate of azo reduction.

Raffii and Cerniglia (1990) developed an anaerobic non-denaturing gel assay for the detection of azoreductase from anaerobic bacteria. They detected azoreductase from *Peptococcus, Eubacterium, C. perfringens* and *Bacteroides.* Their results suggest the presence of only one azoreductase in each of the anaerobic bacteria tested. Except for *C. perfringens* each of the bacteria had only one dehydrogenase, which comigrated with the azoreductase from the same species.

Isolation of the *E. faecalis* azoreductase was unsuccessful despite repeated attempts and following published protocols. It was however possible to characterise the mechanism behind azoreductase activity of *E. faecalis*. Rate of reduction was dependent on:

i) The nature of the reducing agent.

ii) Molecular parameters influencing the ease with which electrons are accepted by the azo group.

For decades azo based dyes have been used in numerous applications. These chemicals represent a potential health hazard as they are widely distributed and some have been shown to be carcinogenic in animal toxicity studies (Shan *et al.*, 1988). Before studies were proceeded any further a characterisation of the study chemical was performed. This was done to assure that the results of the tests were not compromised by unacceptable high levels of impurities in the compound.

Only in recent years has the presence of impurities in synthetic dyes become a matter of great concern to the textile dye chemist. This concern is largely the result of a growing need to conduct biological evaluations of dyes for their potential toxicity, specifically mutagenicity and carcinogenicity. Such evaluations require the use of analytically pure dyes to avoid ambiguities which are likely to arise when interpreting data generated from the use of impure dye samples. Preparative HPLC, dry column chromatography, flash chromatography and countercurrent chromatography have emerged in recent years as very useful procedures for purifying various organic compounds. HPLC required a large volume of solvent for each purification and had very low yield (<1%). Flash chromatography was found to give good purity with higher yield (10%). Due to the small yields from HPLC and TLC, these methods were used to monitor purity of the dye after flash chromatography.

Another approach to the testing of azo dyes was to test their aromatic amine reduction products directly. This approach required that the aromatic amines be commercially available or synthesised. Table 3.7 lists the azo reduction end-products which were either available commercially or were kindly donated by Dr. I. Coutts, Department of Chemistry, The Nottingham Trent University. These aromatic amines were found to be free of coloured products by TLC. They were approximately 90% pure.

4.2 GENOTOXICITY AND MUTAGENICITY

Since many aromatic amines are carcinogenic and mutagenic, a complete evaluation of the safety of azo compounds in the human environment must include an evaluation of the safety of their constituent aromatic amines. If an azo compound is ingested orally, it can be reduced by anaerobic intestinal microflora, and possibly by mammalian azoreductases in the intestinal wall or in the liver, to free aromatic amines (Scheline *et al.,* 1970; Walker, 1970; Gingell and Walker, 1971).

If these aromatic amines are stable, they can be synthesised and tested separately. Many commercial azo compounds, however, contain aromatic amine moieties that are unstable when released by reduction from the azo compounds. This is particularly true when a hydroxyl group is present in an aromatic ring adjacent to a freed amino group, as is the case with azo dyes permitted for use in foods, drugs and cosmetics in both Europe and the United States. Since such aromatic compounds are easily oxidised in air, they are difficult to synthesise in a pure form and test for mutagenicity. Therefore a method is needed for testing the mutagenicity of azo compounds under conditions in which reduction of the compound to free aromatic amines occurs in the presence of both a metabolic activation system and a genetically sensitive indicator organism.

Three azo dyes (amaranth, carmoisine, sunset yellow) that are widely used in foods were non-genotoxic in the differential killing assay, using tester pairs *E. coli* WP2 & CM871 and *E. coli* AB1157 & BH20. They also failed to exhibit mutagenicity in the standard Ames *S. typhimurium* pre-incubation assay, using *S. typhimurium* TA102 and TA104. *E. coli* CM871 is partially deficient in post-replication repair and almost normal in recombination ability and growth. The *Salmonella* tester strain TA102 detects a variety of oxidants and other agents as mutagens which are not detected by the standard tester strains *S. typhimurium* TA98 or TA100. Among the oxidants detected are hydrogen peroxide and other peroxides, X-rays, bleomycin, quinones. TA102 differs from the other standard tester strains used in the mutagenicity screening in that it has A:T base pairs at the site of reversion, whereas all of the other tester strains have G:C base pairs at their reversion sites. It is likely that this difference is responsible for the unique sensitivity of TA102 to reversion by chemical oxidants. Results by Wilcox and colleagues

(1990) showed that the *E. coli* WP2 strains were able to detect a similar range of mutagens as *S. typhimurium* TA102. *S. typhimurium* TA102 and TA104, were added to the standard set TA98 and TA100 as advised by Maron and Ames (1983).

These dyes were also shown by numerous investigators to be non-genotoxic, using a variety of protocols that did not include azo reduction (Brown *et al.*, 1978; Haveland-Smith and Combes, 1980a; Chung *et al.*, 1981). Our experiments extend these previous reports in that we performed the assays following anaerobic pre-incubation involving bacterial, enzymatic and chemical reduction of the dye (Figure 3.7).

After incubation with *E. faecalis* and *B. thetaiotaomicron,* the cleavage products of amaranth and sunset yellow were found to be genotoxic in the differential killing assay using strains *E. coli* WP2 and CM871 (Figure 3.5a & b). When strains *E. coli* AB1157 and BH20 were used no genotoxicity was detected. *E. coli* CM871, *uvr*A *lex*A *rec*A, detects a broad spectrum of mutagens, detecting frameshift mutagens and alkylating agents. Unlike *E. coli* CM871, the fapy-DNA gylcosylase defective strain (*E. coli* BH20) is not unusually sensitive to either gamma-irradiation or uv-irradiation compared with their wild type strain.

There was no change in genotoxicity of amaranth, sunset yellow or carmoisine after a purification step using flash chromatography. This confirms the belief that mutagenicity of these dyes is due to their reduction products and not to any impurities present in the parent compound. There was no apparent relation between the purity of the dyes and their susceptibility to reduction. Rates of reduction for the purified dyes were similar to those of the crude dyes. The availability of analytical data on the purity of three of the dyes (amaranth, sunset yellow and carmoisine) provided additional data on the performance of these systems and permitted an assessment of the relationship between purity and mutagenicity of these compounds.

Results by Garner and Nutman (1977) showed the mutagenicity of direct red 2 was not due to its reduction products, but was due to the presence of a minor coloured impurity. This was detected by TLC. These observations suggest that a reduction product formed from a relatively minor impurity accounts for half or more of the mutagenicity of the sample. The above results indicate

that considerable caution must be exercised in interpreting quantitative data on the mutagenicity of azo dyes - even with partially purified ones - because of the potential for both mutagenic and non-mutagenic contaminants. The mutagenicity of a particular sample may bear little or no relationship to the structure given to the dye.

Following bacterial reduction, the genotoxicity of purified amaranth and sunset yellow correlated well with that of an equimolar amount of end-product that would result from reduction of these dyes (Figure 3.8a & 3.8b). 1-Amino-2-naphthol-3,6-disulphonate and 1-amino-2-naphthol-6-sulphonate (end-products of amaranth and sunset yellow respectively) were genotoxic. The other cleavage products in each case, 1-amino-naphthalene-4-sulphonate and sulphanilic acid respectively were non-mutagenic, suggesting that mutagenicity was due solely to the presence of an amino-naphthol moiety.

Anaerobic azo reduction with washed cell suspensions of either *E. faecalis* or *B. thetaiotaomicron* before mutagenesis testing (Ames test) was also effective in eliciting a positive mutagenic response with amaranth and sunset yellow. Concentrations of 1μg/plate and greater were sufficient in producing a response from reduced amaranth and sunset yellow (Table 3.14a & b). The pre-incubation of the reaction mixture in the absence of agar was also required to detect the mutagenic activity of the reduced azo compounds. It is possible that the reactions required the reagents to be present at higher concentrations than in the plate-incorporation assay, in which reagents are diluted immediately with the 2ml of molten top agar. This mass action affect was shown by Prival and Mitchall (1982) as accounting for the fact that dimethylnitrosamine was mutagenic in a pre-incubation assay using rat liver S9, while this same compound was not mutagenic in a plate incorporation assay with this type of S9.

Amaranth and sunset yellow were activated by *E. faecalis* to a mutagen in *S. typhimurium* TA102 and TA104, but not in *S. typhimurium* TA98 or TA100. The latter is in agreement with the results of numerous investigators who previously studied these dyes (Prival *et al.,* 1988; Ishidate *et al.,* 1994). Testing the amino aromatic portion of both amaranth and sunset yellow resulted in positive mutagenicity in the portion which carried the hydroxy group ortho to the amino group (1-amino-2-naphthol-3,6-disulphonate and 1-amino-2-naphthol-6-sulphonate respectively, Figure 3.12). In

contrast, the naphthalene moieties were found to be non-mutagenic in *S. typhimurium* TA102 and TA104.

Thus reduction of the azo bond was a prerequisite for activation of these dyes to genotoxins and mutagens. The presence of rat liver S9 had no significant effect on mutagenicity of the reduced dyes (Table 3.14). In the differential killing assay, S9 actually decreased genotoxicity. This may be due to inactivation of the azo dye metabolites by reaction with proteins and other substances in S9 or by metabolic inactivation of the reduced dye products. It has been previously shown that hamster S9 is far more effective than rat liver S9 in activating mutagens such as 1-naphthylamine, dimethylnitrosamine, diethylnitrosamine (Prival and Mitchell, 1982). The ability of rat S9 to interfere with the activation of benzidine by hamster S9 implies that detoxification of benzidine or its metabolites is more rapidly catalysed by rat S9 than by hamster S9.

Previous investigators have used dithionite to reduce azo dyes and then tested the resulting solution directly for mutagenicity (Brown *et al.*, 1978; Haveland-Smith and Combes, 1980b; Joachim *et al.*, 1985). This procedure resulted in positive genotoxicity and mutagenicity results for amaranth and sunset yellow in *E. coli* tester pairs WP2 & CM871 and in *S. typhimurium* TA102 and TA104 (Figure 3.7). Carmoisine which was non-genotoxic and non-mutagenic after bacterial azo reduction, was genotoxic after reduction by sodium dithionite (Figure 3.7). While the chemicals used are mild reducing agents and on their own have no effect on the tester strains, it remains to be demonstrated that they do not cause chemical modification other than the type of azo reduction that would occur in the intestine *in vivo*. The reduction of amaranth by sodium dithionite also generated a product more genotoxic than that produced by bacterial reduction. This may be accounted for by examining reduction rate. Reduction was highest for sodium dithionite, followed by *E. faecalis*, followed by stannous chloride and finally *B. thetaiotaomicron* was found to have the slowest reduction rate (Table 3.4). This indicates that the genotoxic agents are unstable and as time prior to testing increases, genotoxicity diminishes.

When an azo compound has a hydroxy group ortho to the azo bond, the resulting aromatic amine reduction product will be readily oxidised in air (Levine, 1991). Thus as time for reduction increases, the reduced end-products become oxidised and genotoxicity decreases. This instability of

the end-products of azo fission and the decrease in genotoxicity was clearly demonstrated. After 24h, the reduced dyes lost genotoxicity (Table 3.6).

The poor chemical stability of the products of azo bond reduction is very important. With the exception of sulphanilic acid and amino-naphthalene, many of the reduction products could not be stored in solution without significant decomposition. For this reason it was preferable to generate azo reduction products from the various test agents chemically or bacterially at the time of the mutagenicity test. In addition since *Salmonella* strains have azoreductase activity (albeit limited), prolonged incubation with the test agents might allow some endogenous reduction products (Brown *et al.*, 1978).

The standard pre-incubation and FMN supplementation protocols were not sufficiently rigorous to demonstrate the potential mutagenicity of amaranth or sunset yellow. Since high concentrations of dye were required to elicit a response in the *Salmonella* pre-incubation assay, these results may simply mean that the dyes are inhibiting their own mutagenicity in the FMN system (Table 3.16a & b). At concentrations used in this study, FMN had very slow azo reduction rates. This lack of mutagenicity cannot be due to the amino-naphthols released upon reduction since positive results were obtained in this system when the aromatic amine alone or bacterial incubation extracts containing these compounds were directly added to this system. Stolz (Dillon *et al.*, 1994) has shown that an azo dye can inhibit the mutagenicity of an arylamine in a dose dependent manner. Prival *et al.* (1988) has shown that amaranth directly interferes with the detection of the mutagenic activity of both the FD&C red No.4 reduction product and N-(2-fluorenyl)acetamide. When a dye is added directly to the S9 activation mixture as in the FMN system, inhibitor(s) could affect the enzymes that metabolise the dye to a mutagen or inactivate the mutagen once formed. Thus the FMN system is a preferable method provided high concentrations are not required for testing that might inhibit mutagenicity and provided FMN is capable of sufficiently reducing azo dyes to their aromatic end-products.

The *Salmonella* FMN pre-incubation method has the advantage of facilitating the testing of azo compounds that have unstable reduction products. This method may fail to detect mutagenicity of reduced azo dyes because the presence of unreduced azo compound may interfere with the

detection of mutagenicity. If it is desired to test the mutagenicity of reduction products in azo dyes, then it may be desirable to employ chemical or bacterial reduction such as those used here.

However, the use of FMN or RF should not be considered as an artificial contrivance to obtain azo reduction, since soluble flavin is likely to be the most normal physiological 'electron shuttle' in azo reduction, being reduced enzymically by NAD(P)H and then reducing azo compounds non-enzymically (Gingell and Walker, 1971; Chung *et al.,* 1978). Bacterial enzymes can mediate the reduction of high molecular weight azo compounds with limited cell permeability if a soluble electron carrier such as FMN is present (Brown, 1981). Chemical reducing agents such as FMN have the advantage of being readily available and of not requiring anaerobic conditions for reduction to occur.

Similarly, following bacterial reduction, Prival and co-workers (1984) found the purified dyes, congo red and direct violet 32, gave the expected number of revertants when compared to equimolar amounts of the parent diamines (benzidine or dimethoxyybenzidine, respectively). In the FMN/hamster system, congo red gave negative dose-response curves at the concentrations chosen for this study (0-1.0μg/plate). Direct violet 32 was essentially negative. However, positive dose response curves have been reported in this system at lower concentrations. These differences may be due to the dyes inhibiting their own mutagenicity in the FMN/hamster system at the higher concentrations. Positive results were obtained when the diamines alone or bacterial incubation extracts containing these compounds were added directly to FMN/hamster system.

Three azo dyes, acid red 85, Acid red 114 and direct blue 53 have been reported as non-mutagenic in *S. typhimurium,* using test protocols in which riboflavin or dithionite were used to effect azo reduction (Brown *et al.,* 1978). A later study by Prival *et al.* (1984) found all three of these dyes to be mutagenic in TA98 in the presence of FMN at concentrations 0.3, 0.1and 0.1 μmoles/plate respectively. This difference may be attributable to Venturini and Tamaro (Prival *et al.,* 1994) employing rat liver S9 and RF as opposed to Prival who used hamster liver S9 and FMN. Prival *et al.* (1994), found the dyes were not mutagenic with rat liver S9 and FMN.

While a separate reduction by faecal bacteria may prevent dyes from inhibiting their own mutagenicity, the FMN/hamster system may yield a greater mutation rate in other circumstances. The most notable example of this is with direct orange 6 (Garner and Nutman, 1977), which is derived from the relatively weak mutagen dimethylbenzidine. Direct orange 6 was largely resistant to reduction in the bacterial/rat system, but it was much more mutagenic than an equimolar amount of dimethylbenzidine in both systems, and was about ten times more potent in FMN/hamster system than in FMN/rat system. Thus it appears that the bacterial/rat system is significantly less sensitive to a mutagen that is either present in this sample of direct orange 6 or that is formed upon reduction. This may be due to incomplete recovery of mutagen from the bacterial incubation, or to decomposition of a chemically reactive mutagen before it is added to the tester strain. The FMN/hamster system, therefore may be the preferable method for detecting mutagenic dye components other than diamines, provided high concentrations are not required that might inhibit mutagenicity. This is an important factor not only for *in vitro* testing, but may be important *in vivo* as well. If a reactive short lived mutagen were formed during reduction by caecal flora, it might combine with intestinal contents before reaching critical tissues of the host animal. For such mutagens, the two step *in vitro* procedure using whole bacteria may be a better model of the *in vivo* events. Thus the different experimental protocols have different strengths and weaknesses that must be considered in relation to the goals of the testing procedure. Thus the overall activity of a particular test system depends not only upon the reductive step but also on the activity of the oxidative enzymes: hamster S9 yields more mutants in the case of benzidine, while Aroclor-induced rat S9 is equal or superior to hamster S9 for the benzidine congeners.

Another approach to the testing of azo compounds is to test the aromatic amine reduction products directly. The lack of specificity of azoreductase implies that any ingested azo compounds are expected to be reduced *in vivo* to free aromatic amines. If negative results are obtained on the azo dye, even in the presence of a reducing agent such as FMN, testing of the aromatic amine reduction products is still important, since the presence of the free azo compound may interfere with the ability to detect the mutagenic activity of the amine. The azo dye amaranth interferes with the mutagenic activity of 2-naphthylamine (Dillon *et al.*, 1994). However this direct approach requires that the aromatic amines be commercially available or synthesised for testing, and also be stable under test conditions. Many of the common commercial azo dyes yield ortho-diamino or ortho-

hydroxyamino compounds as their azo reduction products. The lack of availability and instability of most of these compounds makes a protocol such as the one described here useful, since the unstable aromatic amine is produced directly in the presence of the S9 mix and the bacterial indicator organisms.

Our results show the amino-naphthol end-products of amaranth and sunset yellow (1-amino-2-naphthol-6-sulphonate and 1-amino-2-naphthol-3,6-disulphonate respectively) elicited a positive response in both the differential kill assay (*E. coli* WP2 & CM871, Figure 3.8b & c) and the *Salmonella* pre-incubation assay (*S, typhimurium* TA102 & TA104, Figure 3.12, Table 3.14a & b), despite negative responses in *S. typhimurium* TA98 and TA100.

Experiments by Bonser *et al.* (1956) also demonstrated the carcinogenic activity of the ortho hydroxy-amines 2-amino-1-naphthol, 1-amino-2-naphthol and 3-hydroxy-4-aminodiphenyl. They hypothesised that aromatic amines induce cancer by virtue of their transformation in the body to ortho hydroxy amines. This activity may be due to:

i) direct reaction of the ortho hydroxy amines with the tissue constituents,

ii) conversion of the ortho-hydroxy amines to the true carcinogens by the enzymes of the tissues

It has been shown that 1-phenyl-azo-2-naphthol as well as 1-amino-2-naphthol hydrochloride is carcinogenic to the mouse bladder. It is possible that reduction of 1-phenyl-azo-2-naphthol takes place in the bladder epithelium (Bonser *et al.*, 1956) .

Dillon and co-workers (1994) found 1-amino-2-naphthol to be mutagenic both in the pre-incubation assay (FMN/rat liver S9) and after extraction from rat caecal products, under the same conditions as those resulting in D&C red No. 9 mutagenicity in *S. typhimurium* TA100. Similar concentrations of extracts of the caecal reduction products from D&C red No. 9 and 1-amino-2-naphthol induced similar levels of revertants. It is feasible that in the absence of toxicity, the maximum revertant yields for D&C red No.9 and 1-amino-2-naphthol may have been comparable. It is unlikely that the mutagenicity of reduced D&C red No.9 could be due to formation of 2-amino-5-chloro-4-methylbenzenesulphonic acid (CLT acid) by reduction because this compound is non-mutagenic (Shimizu *et al.*, 1985). 1-amino-2-naphthol was tested up to 200µg/plate in TA100, and the maximum mutagenic affect was detected at 150-160µg/plate.

Both our results and those of Dillon's group (1994) with 1-amino-2-naphthol contrast with two earlier studies in which 1-amino-2-naphthol was reported to be non-mutagenic in *Salmonella.* In one study, 1-amino-2-naphthol was tested in TA1538 to maximum concentration of 100µg/plate (Garner and Nutman, 1977). In the other study, 1-amino-2-naphthol was tested in TA100 and other strains to a maximum concentration of 25µg/ml due to toxicity (Chung *et al.,* 1981). Dillon's group used *S. typhimurium* TA100 with rat liver S9. Our results show 1-amino-2-naphthol to exhibit mutagenicity in *S. typhimurium* TA102 and TA104. These variations indicate the importance of using different *Salmonella* tester strains with and without activation to detect a variety of mutagenic end-points and equally the importance of determining the exact nature of the damage.

In addition to amaranth, sunset yellow and carmoisine, thirteen further dyes were studied for their ability to cause genotoxicity. Those which were genotoxic in the differential kill assay were subsequently assayed in the *Salmonella* pre-incubation mutagenicity assay. Genotoxicity for those dyes with hydroxy groups ortho to the azo linkage increased as number of substituents on the ring decreased (Figure 3.6a,b,c). Orange II and sudan I, with 1-amino-2-naphthol as a common reduction product were both highly genotoxic after azo reduction by *E. faecalis.* The presence of sulphanilic acid (orange I) or benzyl group (sudan I) had no effect on the genotoxicity of these compounds. Orange II and sudan I gave the similar number of revertants when compared with equimolar amounts of the parent, 1-amino-2-naphthol. Bordeau red and amaranth, both produce 1-amino-2-naphthol-3,6-disulphonic acid, and had similar genotoxic activity after azo reduction by *E. faecalis.* The presence of a sulphonate group on the naphthalene ring of amaranth appeared to have no effect on the genotoxicity of this compound. These results compare favourably with those obtained in the *Salmonella* mutagenicity assay using *S. typhimurium* TA102 and TA104 (Table 3.15a & b). Eriochrome blue black, sudan I and orange II were all highly mutagenic after incubation and reduction by *E. faecalis* in TA102 and TA104 (Table 3.15).

Thus among the metabolites or derivatives of azo dyes tested for genotoxicity and mutagenicity, those with the hydroxy group ortho to the amino group were active. For the azo-naphthol dyes studied, as rate of reduction by *E. faecalis* increased the genotoxicity decreased. Whilst amino-naphthols were shown to be non-mutagenic in *S. typhimurium* TA98 and TA100 (Rosenkranz and

Klopman, 1990), when 1-amino-2-naphthol was subjected to CASE analysis it was revealed to have two major biophores which may endow the molecule with mutagenic activity. In the case of 1-amino-2-naphthol it has been shown to be a rodent carcinogen (Garner *et al.*, 1984). Examination of the location of the biophores suggests that every substitutable carbon is covered by one of the biophores and it is to be expected that sulphonation at any position will result in a decreased likelihood of mutagenicity, unless sulphonation results in the formation of new biophores.

A systematic analysis of the consequence of sulphonation of 1-amino-2-naphthol indicated that the destruction of either of these two biophores or both resulted in a decrease or loss of predicted potency (Rosenkranz and Klopman, 1990). Sulphonation at position three or four will result in loss of biophore R2 with a consequent decrease in mutagenicity from 88% to 60%. 1-Amino-naphthalene-4-sulphonic acid is predicted to be marginally active (59.9%). Whether this marginal activity will be realised is of course open to question, especially as Prival *et al.* (1988) reported that the product of chemical reduction of amaranth did not exhibit mutagenicity in the tester strains *S. typhimurium* TA98, TA100, TA1535 and TA1537. Our results using both the *E. coli* (WP2 & CM871) and *S. typhimurium* (TA102 and TA104) assay, also showed negative genotoxicity and mutagenicity.

Chung and colleagues (1981) also found 1-amino-2-naphthol HCl and its 4-sulphonate sodium salt to be toxic to TA98, TA100 and TA1538. The sulphonate derivatives of 4-amino-naphthalene were not mutagenic in any of the tester strains suggesting that ring substitution by the sulphonate groups prevented the activation of the amino group by liver S9.

4.3 GENERATION OF ACTIVE OXYGEN SPECIES

Previous results demonstrated the production of mutagens following azo fission. Mutagenicity was only demonstrated in *Salmonella* strain TA102 and TA104 which detect a variety of oxidants and other DNA-damaging agents. Additionally no genotoxicity was detected when the differential kill assay was carried out under strictly anaerobic conditions. Therefore an

examination of a possible active oxygen (oxygen radical) mechanism of mutagenicity was initiated.

Results by Nakayama and co-workers (1983) showed the production of active oxygen species such as hydrogen peroxide and superoxide radicals from compounds with a labile hydrogen atom in the hydroxyl moiety in the compounds. They detected no active oxygen for compounds which lacked such labile hydrogen. These findings suggest that various azo dye products maybe genotoxic, not through N-hydroxylation and esterification which is characteristic of many aromatic amines (Kimura *et al.*, 1979; Farr and Kogoma, 1991), but rather through a mechanism involving oxygen radicals (Nakayama *et al.*, 1983; Basaga, 1990) (Figure 1.5).

In support of this hypothesis, the production of superoxide radical from reduced azo dyes was detected by the reduction of nitrotetrazolium blue and confirmed using cytochrome C (Table 3.18). The mechanism by which these species are formed is not clear, but, may be specific for ortho-hydroxy aromatic amines and involve the reactions postulated by Nakayama (1983). There may be a requirement for Fe^{2+} , as in Fenton chemistry, where superoxide radicals are converted, via hydrogen peroxide, to the highly reactive hydroxyl species which is known to damage DNA (Basaga, 1990). All the compounds shown to generate active oxygen species had a hydroxyl substituent ortho to the amino function. Superoxide radicals were detected for 1-amino-2-naphthol, 1-amino-2-naphthol-4-sulphonate, 1-amino-2-naphthol-6-sulphonate, 1-amino-2-naphthol-3,6-disulphonate, while no active oxygen was detected for 1-amino-naphthalene-4-sulphonate. Rate of superoxide radical produced was proportional to the genotoxicity of the compound.

Similarly, hydrogen peroxide was only detected from compounds with an hydroxyl group ortho to the amino function (Table 3.19). In a system where the two types of active oxygen species are formed, i.e. hydrogen peroxide and superoxide radicals, further reaction leading to the generation of a more reactive species of free radical, hydroxyl radical, is expected via the Haber-Weiss reaction,

$$H_2O_2 + O_2^{\cdot-} \rightarrow OH^{\cdot} + OH^{-} + O_2$$

This reaction has been found to be progressively enhanced in the presence of iron ions (Halliwell, 1987).

In accordance with this theory, it was shown that superoxide radicals generated using xanthine/ xanthine oxidase were genotoxic in the differential kill assay using *E. coli* strains WP2 & CM871 and not in *E. coli* strains AB1157 & BH20 (Table 3.11). The free radical generated also exhibited mutagenicity in *S. typhimurium* TA102 and TA104, pre-incubation assay (Table 3.17). Hydrogen peroxide was also shown to elicit a positive response in both these systems (Table 3.12) .

Since oxygen radicals were predicted to be involved behind the genotoxicity of azo dyes, the affect of chemical antioxidants on the genotoxicity of the reduced dyes and their predicted end-products was examined. Antioxidants can act at different levels in the oxidative sequence. They can act by:

i) Decreasing localised oxygen concentrations.
ii) Preventing first chain initiation by scavenging initiating radicals such as hydroxyl radical.
iii) Binding metal ions in forms that will not generate such initiating species as hydroxyl radical, ferryl, or Fe^{2+} /Fe^{3+} /O_2 and/or will not decompose lipid peroxides to peroxy or alkoxy radicals.
iv) Decomposing peroxides by converting them to non-radical products such as alcohols.
v) Chain breaking, scavenging intermediate radicals such as peroxy and alkoxy radicals to prevent continued hydrogen abstraction.

Ascorbic acid present in high concentrations protected *E. coli* cells from damage by reduced azo dyes and their predicted amino-naphthol end-products (Figure 3.9). Ascorbic acid can act as a reducing agent (electron donor). Its ability to reduce Fe(III) to Fe(II) is important in promoting the uptake of iron in the gut (Halliwell and Gutteridge, 1986). Ascorbate reacts rapidly with $O_2^{\cdot-}$ and HO_2' and even more rapidly with OH' to give semi-dehydroascorbate. It also scavenges singlet oxygen. Hence ascorbate may well help to protect against oxygen-derived species *in vivo.* A further antioxidant activity of ascorbate is its recycling of α-tocopherol. However, ascorbate can also be pro-oxidant. Like $O_2^{\cdot-}$, ascorbate can reduce Fe(III) ions to Fe(II) and in the presence of hydrogen peroxide, can stimulate OH' formation by the Fenton reaction. Its overall effect will depend on the concentration of ascorbate present, since it also scavenges OH'. Ascorbate and copper rapidly inactivates the hydrogen peroxide degrading enzyme, catalase and several authors have described

cytotoxic and mutagenic effects of ascorbate on isolated cells, probably involving its interaction with iron and /or copper ions added to the cell suspension media (Halliwell and Gutteridge, 1990).

α-Tocopherol, found in membranes and lipoproteins in the body, blocks the chain reaction of lipid peroxidation by scavenging intermediate peroxyl radicals. It provided good protection against damage by the reduced azo dyes and their amino-naphthol end-products (Figure 3.9). α-Tocopherol is oxidised by superoxide-generating systems (probably by reaction with HO_2 '). α-Tocopherol also reacts with OH' at an almost diffusion-controlled rate.

Retinoic acid is a lipid soluble antioxidant, but it does not have major chain breaking activity. It was capable of providing protection against damage by reduced dyes. The dietary antioxidants l-ergothioneine and chlorophyllin were both capable of protecting *E. coli* from damage by reduced dyes and their amino-naphthol end-products. This suggests that both l-ergothioneine and chlorophyllin can act as either scavengers of hydroxyl radicals or as inhibitors of their formation at the concentrations tested. This result is confirmed by Hartman (1990) who showed singlet oxygen was eliminated by l-ergothioneine and further confirmed its antioxidant properties.

Caffeic acid was also tested in this system and was found to be genotoxic in its own right. This was not unexpected when one considers results obtained by Inoue and co-workers (1978). They found that in the presence of Mn(II) or Cu(II), caffeic acid produced hydrogen peroxide. The enhancing effects of 3-amino-triazole and the inhibitory effect of catalase on caffeic acid induced DNA damage all indicate the participation of hydrogen peroxide.

BHA and BHT, two of the most controversial antioxidants, were also studied for their protective effects against reduced azo dye induced damage (Table 3.10). Both caused a decrease in viability of *E. coli* WP2 (repair proficient). The addition of *E. faecalis* reduced amaranth enhanced genotoxicity. BHA has been shown to produce hyperplasia and/or tumours in the forestomach of rats, mice and hamsters. However, it was concluded that the production of these tumours was not a manifestation of genotoxicity (Poulsen, 1991). BHA has been shown to be converted metabolically to quinones that interact with the P-450 part of the hepatic mono-oxygenase system to form hydrogen peroxide (Clayson *et al.*, 1994). At low levels, hydrogen peroxide is efficiently degraded

by enzymes such as catalase. Phillips *et al.* (1987) showed that, *in vitro,* BHA was clastogenic if catalase and related enzymes were stripped from the culture medium and from the microsomal fraction to induce metabolism. A recent study by Schilderman and colleagues (1995) showed BHA to be a strong inducer of oxidative DNA damage in the epithelial cells of the glandular stomach of rats, increasing the level of 8-OHdG with increasing duration of BHA administration.

The data indicate the involvement of major oxidants in inducing DNA damage by reduced azo dyes (Figure 3.10a & b). It also suggests that the active oxygen species include superoxide radical and hydrogen peroxide. The complete inhibition of genotoxicity by catalase and partial inhibition by superoxide dismutase reflects a possible contribution of hydrogen peroxide via Fenton type reactions in the damage caused by these compounds (Table 3.12). Cellular catalase appeared to play a major role in preventing reduced azo dye damage in *E. coli.* Inhibition of cellular catalase by 3-amino-triazole led to an increase in, and nearly equal susceptibility to, cell damage in *E. coli* WP2 and CM871. The addition of extracellular catalase to this system appeared to decrease the genotoxicity of reduced dyes (Figure 3.10a & b).

SOD, which did not provide complete protection in our system (Table 3.13), has been previously shown to protect DNA strand breaks in isolated DNA exposed to xanthine oxidase/xanthine (Mashino and Fridovich, 1987), presumably by preventing the superoxide-induced reduction of Fe(III) to Fe(II) which in the presence of hydrogen peroxide reforms Fe(III) + OH' + OH^-. A number of cellular reductants, e.g. ascorbate, may reduce Fe(III) inside whole cells so that superoxide may not be essential to augment the formation of OH' within cells.

Typical OH' scavengers, sodium benzoate, mannitol and potassium iodide, provided none or little protection against damage by reduced dyes and amino-naphthols (Table 3.13). Since OH' reacts indiscriminately with any molecule and has a diffusion radius of 2.3nm (Hutchinson, 1985), one may assume that the OH' scavengers never reached the site of OH' attack right at the DNA. Since the rate constant for the reaction of various OH' scavengers is of the same order of magnitude as that of OH' with deoxyribose, and since nucleotides are present in molar concentrations in the nucleosome, it is not surprising the OH' scavengers could not compete efficiently in this reaction.

The reaction between $O_2^{\cdot-}$ and hydrogen peroxide to give OH' does not occur unless traces of iron salts are present in the system (Table 3.13) (Gutteridge *et al.,* 1979; McCord and Day, 1978; Halliwell, 1987). This iron-dependent generation of OH', the Fenton or Haber-Weiss reaction, can be inhibited by metal-ion chelators, such as desferrioxamine (Table 3.13). If this reaction is the mechanism behind genotoxicity of reduced azo dyes, the presence of metal chelators should therefore inhibit the reaction. Figure 4.2 is a schematic representation of the possible mechanism behind genotoxicity of 1-amino-2-naphthol-6-sulphonate, the predicted end-product of sunset yellow. The lack of protection by EDTA does not exclude the genotoxicity of reduced azo dyes being mediated by a Fenton type reaction. EDTA has been shown to either stimulate or inhibit iron-mediated lipid peroxidation (Halliwell and Gutteridge, 1990), depending on the ratio of the concentration of chelator to that of iron salt. Iron ions complexed with EDTA are still capable of forming OH' from $O_2^{\cdot-}$ and hydrogen peroxide. Fe(III)-EDTA is reduced by $O_2^{\cdot-}$ and Fe(II)-EDTA is oxidised by hydrogen peroxide leading to OH' formation. It can sometimes protect by preventing site specific reactions, if iron ions are bound to a specific critical target that is destroyed by OH', then EDTA can protect by withdrawing iron ions from that site. The Fe-EDTA complex will generate OH' in free solution, but less OH' will reach the target.

Both *o*-phenanthroline and desferrioxamine provided protection against damage by reduced azo dyes and their amino-naphthol end-products (Table 3.13). Complexes of iron salts with o-phenanthroline and desferrioxamine have been shown by Halliwell and Gutteridge (1990) to diminish formation of OH' from $O_2^{\cdot-}$ and hydrogen peroxide. The substance o-phenanthroline, which penetrates easily into cells, has been shown to diminish hydrogen peroxide-dependent damage in cells, perhaps by binding iron ions from the vicinity of the DNA and preventing DNA fragmentation by site specific OH' generation. Desferrioxamine is now the most widely used chelator of iron. It is highly specific for iron which it binds as Fe(III). The Fe(III)-desferrioxamine complex (ferrioxamine) is very difficult to reduce, not only by $O_2^{\cdot-}$, but also by more powerful reductants.

Thus oxidative DNA damage by reduced dyes was dependent upon:

i) the level of DNA-damaging agent,

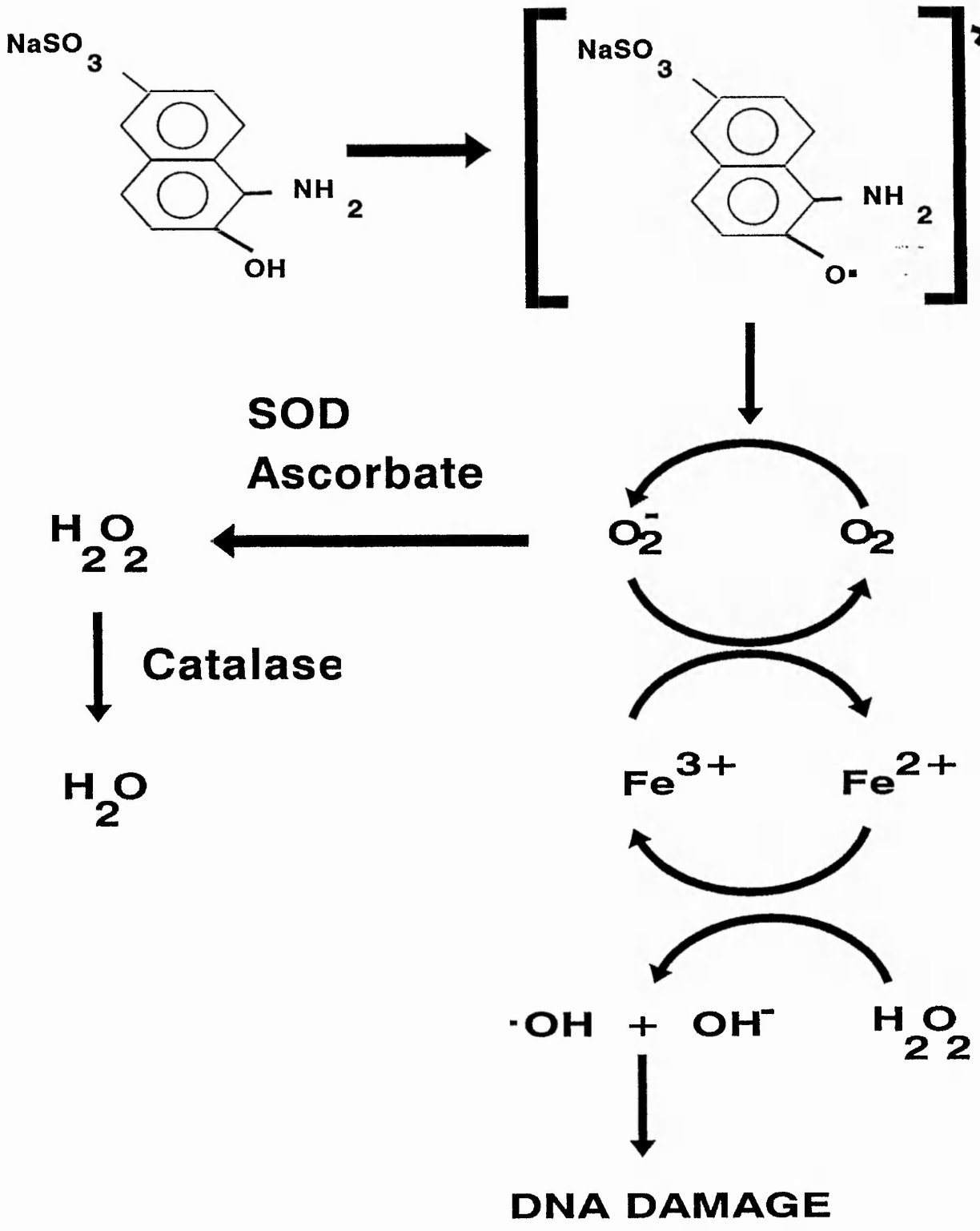


Figure 4.2 The generation of active oxygen species from aminonaphthols

* intermediate proposed by Nakayama et *al.* 1983.

ii) the rate of decomposition of hydrogen peroxide by catalase, superoxide dismutase and other antioxidant enzymes,

iii) the availability of transition metal ions proximate to the DNA.

Hydroxyl radicals react with the DNA bases at diffusion-controlled rates by addition reactions. For example, OH' can add on to guanine at C-4, C-5 and C-8 positions to give OH-adduct radicals (Halliwell and Dizdaroglu, 1992). Addition of OH' at position C-8 of guanine produces a C-8 OH-adduct radical that can be reduced to 8-hydroxy-7,-8-dihydroguanine, oxidised to 8-hydroxyguanine (8-OHGua) or undergo opening of the imidazole ring, followed by one-electron reduction and protonation to give 2,6-diamino-4-hydroxy-5-formamidopyrimidine, usually abbreviated as FapyGua.

8-Hydroxyguanine is one of the major products of base damage when DNA is exposed to physiologically-relevant systems producing OH'. In the ferric ion/hypoxanthine/xanthine oxidase system and chelate/H_2O_2 system (± ascorbate) 8-OHGua represents over 30% of the total base modification products measured (Arouma *et al.*, 1989). 8-Hydroxyguanine (8-OHGua) describes the purine base guanine in which the H atom at position 8 is replaced by an OH group. It can be released from DNA by acidic hydrolysis. If enzymic hydrolysis is used, 8-OHGua may be released still attached to the deoxyribose sugar. This product is called 8-hydroxy-2-deoxyguanosine (8-OHdG). The development of an HPLC-based technique coupled with highly-sensitive electrochemical detection, for the measurement of 8-OHdG released from DNA by enzymic digestion was the main impetus that led us to look for this product after incubation of calf thymus DNA with reduced azo dyes and amino-naphthols.

Levels of 8-OHdG increased in both calf thymus DNA and *E. coli* BH20 DNA after pre-treatment with hydrogen peroxide (Figure 3.18, Table 3.21). Blakely and co-workers (1990) also showed an increase in 8-OHGua levels after aqueous solutions of calf thymus DNA were exposed to hydrogen peroxide. Formation of 8-OHdG in calf thymus DNA and *E. coli* BH20 was also shown after exposure to reduced amaranth and 1-amino-2-naphthol-3,6-disulphonate, its predicted end-product. The yield of this product was dependent on the reduced dye or amino-naphthol concentration (Table 3.22).

Further evidence to support the involvement of hydrogen peroxide and hydroxyl radicals in damage caused by reduced azo dyes and the subsequent detection of the DNA base product of 8-OHdG comes from the inhibition of product formation by catalase (Figure 3.22). Hence we propose that in the systems used in the present study, the mechanism behind genotoxicity of reduced dyes is the formation of hydroxyl radical, via a hydrogen peroxide and $O_2^{\cdot-}$ assisted Fenton reaction. Superoxide dismutase, catalase, desferrioxamine all protect against genotoxicity.

The appearance of increased levels of 8-OHdG after pre-incubation with reduced dyes in *E. coli* BH20 (Figure 3.21) may be explained by the fact that this strain of *E. coli* is defective in formamidopyrimidine-DNA-glycosylase (Fpg protein). The Fpg protein has been shown to have the ability to excise 8-OHGua residues from methylene-blue-light treated DNA (Czeczot *et al.*, 1991). Since the ultimately reacting species generated by MB-light is most likely singlet oxygen, Fpg protein should be considered as a protein protecting DNA from the deleterious effects of reactive oxygen species as well as those of alkylating agents (Boiteux *et al.*, 1989; Breimer, 1990; Czeczot *et al.*, 1991). Chung and colleagues (1991) described an endonuclease of *E. coli*, that specifically removed 8-OHGua residues from DNA. Fapy glycosylase removes the imidazole ring open form of purines, particularly Fapy produced from a 7-methylguanine residue by alkaline treatment (Breimer and Lindahl, 1984).

The cleavage sites of Fapy glycosylase are the same as that of 8-OHGua endonuclease (O'Connor and Laval, 1989). The 8-OHGua endonuclease showed negligible activity against Fapy-containing DNA (Chung *et al.*, 1991). There is a possibility that the 2 enzymes are the same and show much higher activity toward 8-OHGua containing DNA than Fapy-containing DNA. The inability of the reduced dyes to elicit a genotoxic response in this strain of *E. coli* may be due to other protective systems within the cells. The presence of 8-OHdG is only a marker of oxidative DNA damage, but is not necessarily what is causing cell death.

4.4 CONCLUSION

Exposure of *E. coli* to reduced azo dyes or to their amino-naphthol end-products results in DNA damage that causes cell death. Auto-oxidation of active metabolites of reduced dyes and amino-naphthols generated hydrogen peroxide and superoxide anion. Catalase and SOD were used to identify the formation of these active oxygens. In a system where the two types of active oxygens are formed, further reaction leading to the generation of a more reactive species of free radical, OH radical, is expected *via* the Haber-Weiss reaction,

$$H_2O_2 + O_2^{\cdot-} \rightarrow OH' + O_2^{\cdot-} + O_2$$

Iron is extremely efficient at generating OH' by the above reaction, and the complete reaction is as follows:

$$\text{Fe(III) - complex} + O_2^{\cdot-} \rightarrow \text{Fe(II)- complex} + O_2$$

$$\text{Fe(II) - complex} + H_2O_2 \rightarrow OH' + O_2^{\cdot-} + \text{Fe(III) - complex}$$

$$O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + OH' + O_2^{\cdot-}$$

the iron-catalysed Haber-Weiss reaction

Iron complexes able to catalyse OH' formation by this mechanism *in vivo* seem to be provided by the low molecular mass intracellular iron pool, and by the ability of $O_2^{\cdot-}$ and hydrogen peroxide to release catalytically-active iron ions from iron proteins. For example $O_2^{\cdot-}$ can release iron ions from ferritin, whereas hydrogen peroxide releases iron from haemoglobin. Thus increased generation of $O_2^{\cdot-}$ and hydrogen peroxide can create the conditions that lead to OH' formation. Hydroxyl radicals, once generated, react with the molecules of their immediate surroundings. The much lower reactivity of $O_2^{\cdot-}$ and hydrogen peroxide means that they can diffuse away from their sites of formation, leading to OH' generation in different parts of the cell whenever they meet a 'spare' iron ion. Hence the toxicity of $O_2^{\cdot-}$ and hydrogen peroxide to cells is influenced by the intracellular distribution of metal ions, and sequestration of metal ions in 'safe' forms contributes to antioxidant defence mechanism. The protective effects of

desferrioxamine and o-phenanthroline against damage by reduced dyes and amino-naphthols indicates the importance of iron in the mechanism behind their toxicity. Protection by antioxidants also indicates the involvement of active oxygen species in the genotoxicity of these compounds.

Our proposed method of oxidative damage from reduced dyes and their amino-naphthol derivatives is not unexpected when one considers a study by Tagesson *et al.* (1993). They found levels of 8-OHdG in urinary samples of azo-dye workers to be significantly higher than in control groups. Their analysis could find no important association between 8-OHdG excretion, age and smoking, suggesting that occupational exposures contributed to the increase in oxidative damage to human DNA.

In recent years there has been increasing interest in the reactions of free radicals, especially oxygen radicals, in promoting tumour production. It is particularly important to understand the reactions of free radicals and to determine the mechanism of damage involved. Understanding the mechanism of damage caused by reduced azo dyes may lead to a better control of the systems that produce radicals and provide knowledge useful for designing new azo dyes.

Chemical carcinogens can be sorted into three general categories (Zeiger, 1993), those that contain or can form electrophilic centers, and are thereby capable of directly reacting with DNA; those that do not directly react with DNA, but can produce secondary products that can react with or damage DNA; and those that do not mutagenise or damage DNA directly or indirectly, but modify or moderate normal cell functions. Because radical generators are mutagenic through the secondary generation of free radicals, they should be considered a different category of mutagens than those that act via a direct electrophilic reaction with DNA.

Questions surrounding the free-radical inducing class of chemicals relate to:

i.) the likelihood that the reactions observed *in vitro* also take place *in vivo*,

ii.) the extent to which the addition of these chemicals to food increases the levels over background,

iii.) the production of free radicals at or adjacent to the cell's DNA.

Although many food additives are capable of producing free radicals, many can also act as antioxidants. Free- and oxygen radical scavenging mechanisms also exist in the body, whereas they are generally not present in the *in vitro* mutagenesis tests. It is also not known to what extent these free radical reactions occur *in vivo* at physiological concentrations of reactants and free-radical scavengers. The continuous threat of oxidant damage to the cell, tissue and organism as a whole is underscored by the existence of an impressive array of cellular defences that have evolved to battle these reactive oxidants. The oxidative damage rate in mammalian species such as rats, with a high metabolic rate, short life-span is actually higher than the rate in humans who have lower metabolic rates and longer life-spans. Thus the ability of reduced dyes to generate oxygen radicals may not pose as a threat to the human populations. However it is important to reassess the ability of toxicology tests to detect this type of damage.

BIBLIOGRAPHY

BIBLIOGRAPHY

Aiyar, J., K.M. Borges, R.A. Floyd and K.E. Wetterhahn. 1989. Role of chromium(V), glutathione thiyl radical and hydroxyl radical intermediates in chromium(VI)-induced DNA damage. Toxicol. Environ. Chem. **22**:135-148.

Allen, R.J. and J.J. Roxon. 1974. Metabolism by intestinal bacteria: the effect of bile salts on tartrazine azo reduction. Xenobiotica **4:**637.

Ames, B.N. 1976. The detection of carcinogens as mutagens: the Salmonella/microsome test, p. 57-62. *In* F.J. de Serre, J.R. Fouts, J.R. Bend, and R.M. Philpot (ed.), In vitro Activation in Mutagenesis Testing, Elsevier, Amsterdam.

Ames, B.N. 1989. Endogenous DNA damage as related to cancer and ageing. Mutat Res. **214**:41-46.

Ames, B.N., J. McCann, and E. Yamasaki. 1975. Methods for detecting carcinogens and mutagens with salmonella/mammalian microsome mutagenicity test. Mutat. Res. **31**:347-364.

Anderson, M.E. and A. Meister. 1983. Transport and direct utilisation of gamma-glutamylcyst<e>ine for glutathione synthesis. Proc. Natl. Acad. Sci. USA. **80**:707-711.

Anderson, M.E., Powrie, F., Puri, R.N. and A. Meister. 1985. Glutathione monoethyl ester: preparation, uptake by tissues, and conversion to glutathione. Arch. Biochem. Biophys. **239**:538-548.

Aruoma, O.I., B. Halliwell, E. Gajewski and M. Dizdaroglu. 1989. Damage to the bases in DNA induced by hydrogen peroxide and ferric ion chelates. J. Biolog. Chem. **264**:20509-20512.

Aruoma, O.I., B. Halliwell, E. Gajewski and M. Dizdaroglu. 1991. Copper-ion-dependent damage to the bases in DNA in the presence of hydrogen peroxide. Biochem. J. **273**:601-604.

Aruoma, O.I. 1994. Nutrition and health aspects of free radicals and antioxidants. Food Chem. Toxicol. **32**:671-683.

Aruoma, O.I. and B. Halliwell. 1991. DNA damage and free radicals. Chem. Br. **2**:149-152.

Aruoma, O.I., B. Halliwell and M. Dizdaroglu. 1989. Iron ion-dependent modification of bases in DNA by the superoxide radical-generating system hypoxanthine/xanthine oxidase. J. Biol. Chem. **264**:13024-13028.

Ashby, J. 1992. Consideration of CASE predictions of genotoxic carcinogenesis for omeprazole, methapyrilene and azathioprine. Mutat. Res. **272**:1-7.

Ashby, J., P.A. Lefevre, B. Burlinson and M.G. Penman. 1985. An assessment of the *in vivo* rat hepatocyte DNA-repair assay. Mutat. Res. **156**:1-18.

Ashby, J., D. Paton, P.A. Lefevre and J.A. Styles. 1982. Evaluation of two suggested methods of deactivating organic carcinogens by molecular modification. Carcinogenesis **3**:1277-1282

Ashby, J., J. Styles and R.D. Callander. 1980. Comparative activity of 4,4'-diaminobiphenyl (benzidine) and its terphenyl analogues, 4,4'-diaminoterphenyl, in two *in vitro* assays for potential carcinogenicity. Carcinogenesis **1**:801-802.

Bachmann, B.J. 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriological Reviews **36**:525-557.

Bachmann, B.J. 1990. Linkage map of *Escherichia coli* K-12, Edition 8, Microbiology Reviews **54**:130-197.

Bagchi, D., E.A. Hassoun, M. Bagchi and S.J. Stohs. 1993. Protective effects of antioxidants against endrin-induced hepatic lipid peroxidation, DNA damage, and excretion of urinary lipid metabolites. Free Radical Biol. Med. **15**:217-222.

Bailly, V., W.G. Verly, T. O'Connor and J. Laval. 1989. Mechanism of DNA strand nicking at apurinic/apyrimidinic sites by *Escherichia coli* [formamidopyrimidine]DNA glycosylase. Biochem. J. **262**:581-589.

Barrueco, C. and E. de la Pena. 1988. Mutagenic evaluation of the pesticides captan, folpet, captafol, dichlorofluanid and related compounds with the mutants TA102 and TA104 of *Salmonella typhimurium.* Mutagenesis **3**:467-480.

Bartsch, H., A. Barbin, M.-J. Marion, J. Nair and Y. Guichard. 1994. Formation, detection, and role in carcinogenesis of ethenobases in DNA. Drug Metab. Revs. **26**:349-371.

Bartsch, H., C. Malaveille, A. Barbin and G. Planche. 1979. Mutagenic and alkylating metabolites of halo-ethylenes, chlorobutadienes and dichlorobutenes produced by rodent or human liver tissues. Evidence for oxirane formation by P-450-linked microsomal monooxygenase. Arch. Toxicol. **41**:249-277.

Basaga, H.S. 1990. Biochemical aspects of free radicals. Biochem. Cell Biol. **68**:989-998.

Beauchamp, C. and I. Fridovich. 1970. A mechanism for the production of ethylene from methional. The generation of the hydroxyl radical by xanthine oxidase. J. Biol. Chem. **245**:4641-4646.

Beckman, J.S., T.W. Beckman, J. Chen, P.A. Marshall and B.A. Freeman. 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc. Natl. Acad. Sci. USA **87**:1620-1624.

Belser, W.L., S.D. Shaffer, R.D. Bliss, P.M. Hynds, L. Yamamoto, J.N. Pitts and J.A. Winer. 1981. A standardised procedure for quantification of the Ames salmonella/mammalian-microsome mutagenicity test. Environ. Mutagen. **3**:123-139.

Bendich, A., E. Gabriel, L.J. Machlin. 1983. effect of dietary level of vitamin E in the immune system of the spontaneously hypertensive and normotensive Wistar Kyoto rat. J. Nutr. **113**:1920-1926.

Berglin, E.H. and J. Carlsson. 1986. Effect of hydrogen sulphide on the mutagenicity of hydrogen peroxide in *Salmonella typhimurium* strain TA102. Mutat. Res. **175:**5-9.

Bielski, B.H.J. 1985. Reactivity of HO_2/O_2^- radicals in aqueous solution. Phys. Chem. Ref. Data **14**:1041-1045.

Bielski, B.H.J., and G.G. Shiue. 1978. Reaction rates of superoxide radicals with the essential amino acids, p43-56. *In* D.W. Fitzsimons (ed.), Oxygen free radicals and tissue damage. Elsevier Science Publishing, Inc., New York.

Blakely, W.F, A.F. Fuciarelli, B.J. Wegher and M. Dizdaroglu. 1990. Hydrogen peroxide-induced base damage in deoxyribonucleic acid. Radiation Res. **121**:338-343.

Boiteux, S., E. Gajewski, J. Laval and M. Dizdaroglu. 1992. Substrate specificity of the *Escherichia coli* Fpg protein (formamidopyrimidine-DNA glycosylase): Excision of purine lesions in DNA produced by ionising radiation or photosensitization. Biochem. **31**:106-110.

Boiteux, S. and O. Huisman. 1989. Isolation of a formamidopyrimidine-DNA glycosylase (*fpg*) mutant of *Escherichia coli* K12. Mol. Gen. Genet. **215**:300-305.

Boiteux, S., T.R. O'Connor and J. Laval. 1987. Formamidopyrimidine-DNA glycosylase of *Escherichia coli:* cloning and sequencing of the *fpg* structural gene and overproduction of the protein. EMBO J. **6**:3177-3183.

Boiteux, S., T.R. O'Connor, F. Lederer, A. Gouyette and J. Laval. 1990. Homogenous *Escherichia coli* Fpg protein. A DNA glycosylase which excises imidazole ring-opened purines and nicks DNA at apurinic/apyrimidinic sites. J. Biol. Chem. **265**:3916-3922.

Bonser, G.M., L. Bradshaw, D.B. Clayson, J.W. Jull. 1956. A further study of the carcinogenic properties of ortho-hydroxy-amines and related compounds of bladder implantation in the mouse. Br. J. Cancer **10**:539.

Borg, D.C. and K.M. Schaich. 1984. Cytotoxicity from coupled redox cycling of auto-oxidising xenobiotics and metals. Isr. J. Chem. **24**:38.

Borg, D.C. and K.M. Schaich. 1988. Pro-oxidant action of antioxidants. In Miguel, J., A.T. Quintanilha and H.Weber (Eds.) CRC handbook of free radicals and antioxidants in biomedicine. Vol 1. Boca Raton, FL. CRC Press Inc. pp 63-80.

Bos, R.P., J.M. Neis, P.J.L. van Gemert and P.Th. Henderson. 1983. Mutagenicity testing with the salmonella/hepatocyte and the salmonella/microsome assays. Mutat. Res. **124**:103-112.

Brawn, K. and I. Fridovich. 1981. DNA strand scission by enzymically generated oxygen radicals. Arch. Biochem. Biophys. **206**:414-419.

Breimer, L.H. 1990. Molecular mechanisms of oxygen radical carcinogenesis and mutagenesis: The role of DNA base damage. Mol. Carcinogenesis **3**:188-197.

Breimer, L.H. and T. Lindahl. 1984. Excision of oxidides thymine from DNA. J. Biol. Chem. **259**:5543-5548.

Bridges , B.A. 1976. Short term screening tests for carcinogens. Nature **261**:195-200.

Bridges , B.A., R.P. Mottershead, M.A. Rothwell and M.H.L Green. 1972. Repair-deficient bacterial strains suitable for mutagenicity screening: Tests with the fungicide captan. Chem. Biol. Interactions. **5**:77-84.

Brown, J.P. 1981. Reduction of polymeric azo and nitro dyes by intestinal bacteria. Appl. Environ. Microbiol. **41**:1283-1286.

Brown J.P. and P.S. Dietrich. 1983. Mutagenicity of selected sulphonated azo dyes in the salmonella/microsome assay: Use of aerobic and anaerobic activation procedures. **116**:305-315.

Brown, J.P., G.W. Roehm and R.J. Brown. 1978. Mutagenicity testing of certified food colours and related azo, xanthene and triphenylmethane dyes with the salmonella/microsome system. Mutat. Res. **56**:249-271.

Brusick, D.J. 1987. Implications if treatment-conditioned-induced genotoxicity for chemical screening and data interpretation. Mutat. Res. **189**:1-6.

Burkitt, P.O. 1976. A deficiency of dietary fibre may be one cause of certain colonic and venous disorders. Digestive Dis. **21:**104.

Burton, G.W., S.C. Cheng, A. Webb and K.U. Ingold. 1986. Vitamin E in young and old human red blood cells. Biochim. Biophys. Acta. **860**:84-90.

Burton, G.W., Y. Le Page, E.J. Gabe, K.U. Ingold. 1980. Antioxidant activity of vitamin E and related phenols. Importance of stereoelectronic factors. J. Am. Chem. Soc. **102**:7791-7792.

Butterworth, B.E. 1989. Nongenotoxic carcinogens in the regulatory environment. Regul. Toxicol. Pharmacol. **9**:244-256.

Butterworth, B.E., E. Bermudez, T. Smith-Oliver, L. Earle, R. Cattley, J. Martin, J.A. Popp, S. Strom, R. Jirtle and G. Michalopoulos. 1984. Lack of genotoxic activity of di(2-ethylhexyl)phthalate in rat and human hepatocytes. Carcinogenesis **5**:1329-1325.

Byrd, S., D. Reines and P.W. Doetsch. 1990. Effects of oxidative DNA damage on transcription by RNA polymerases. Free Radic. Biol. Med. **9**:47.

Cabera, M., Y. Nghiem and J.H. Miller. 1988. *MutM*, a second mutator locus in *Escherichia coli* that generates G.C → T.A transversions. J. Bacteriol. **170**:5405-5407.

Calderaro, M., E.A. Martins and R. Meneghini. 1993. Oxidative stress by menadione affects cellular copper and iron homeostasis. Mol. Cell. Biochem. **126**:17-23.

Cameron, E. 1982. Vitamin C and cancer: an overview. Int. J. Vitam. Nutr. Res. Suppl. **23**:115-127.

Carlsson, J., E.H. Berglin, R. Claesson, M.B. Edlund and S. Persson. 1988. Catalase inhibition by sulphide and hydrogen peroxide induced mutagenicity in *Salmonella typhimurium* strain TA102. Mutat. Res. **202**:59-64.

Cerniglia, C.E., J.P. Freeman, W. Franklin and L.D. Pack. 1982. Metabolism of benzidine and benzidine conger based dyes by human, monkey and rat intestinal bacteria. Biochem. Biophys. Re. Commun. **107**:1224-1229.

Cerniglia, C.E., Z. Zhuo, B.W. Manning, T.W. Federle and R.H. Heflich. 1986. Mutagenic activation of the benzidine-based dye direct black 38 by human intestinal microflora. Mutat. Res. **175**:11-16.

Chambers, R.W. 1985. Chemical carcinogenesis: a biochemical overview. Clin. Biochem. **18**:158-168.

Chambers, D.E., D.A. Parks, G. Patterson, R.S. Roy, J.M.McCord, S. Yoshida, L. Parmley and J.M. Downey. 1985. Xanthine oxidase as a source of free radical in myocardial ischemia. J. Mol. Cell. Cardiol. **17**:145-152.

Chan, A., D. Harbin and C.W. Jameson. 1988. Analyses of two azo dyes by high-performance liquid chromatography. J. Chromatogr. Sci. **26**:439-442.

Chan E. and B. Weiss. 1987. Endonuclease IV of *Escherichia coli* is induced by paraquat. Proc. Natl. Acad. Sci. USA. **84**:3189-3193.

Cheng, K.C., D.S. Cahill, H. Kasai, S. Nishimura and L. Loeb. 1992. 8-Hydroxyguanine, an abundant form of DNA damage causes G---T and A---C substitutions. J. Biol. Chem. **267**:166-172.

Cheng, Y.G. and N.D. Chasteen. 1991. Role of phosphate in initial iron deposition in apoferritin. Biochemistry **30**:2947-2953.

Chesis, P.L., D.E. Levin, M.T. Smith, L. Ernster and B.N. Ames. 1984. Mutagenicity of quinones: pathways of metabolic activation and detoxification. Proc. Natl. Acad. Sci. USA **81**:1696-1700.

Chetsanga, C.J. and T. Lindahl. 1979. Release of 7-methylguanine whose imidazole ring has been opened from damaged DNA by a DNA glycosylase from *Escherichia coli*. Nucleic Acid Res. **6**:3673-3683.

Childs, J.J., C. Nakajima and D.B. Clayson. 1967. The metabolism of 1-phenylazo-2-naphthol in the rat with reference to the action of the intestinal flora. Biochem. Pharmac. **16**:1555.

Chung, K-T. 1983. The significance of azo reduction in the mutagenesis and carcinogenesis of azo dyes. Mutat. Res. **114**:269.

Chung, K-T. and C.E. Cerniglia. 1992. Mutagenicity of azo dyes: structure-activity relationships. Mutat. Res. **277**:201-220.

Chung, K-T., G.E. Fulk and A.W. Andrews. 1978. The mutagenicity of methyl orange and metabolites produced by intestinal anaerobes. Mutat. Res. **58**:375-379.

Chung, K-T, G.E. Fulk and A.W. Andrews. 1981. Mutagenicity testing of some commonly used dyes. Appl. Environ. Microbiol. **42**:641-648.

Chung, K.-T., G.E. Fulk and M. Egan. 1978. Reduction of azo dye by intestinal anaerobes. Appl. Environ. Microbiol. **35**:558.

Chung, K-T and S.E. Stevens. 1992. The reduction of azo dyes by the intestinal microflora. Crit. Rev. Microbiol. **18**:175-190.

Chung, M.H., H. Kasai, D.S. Jones, H. Inoue, H. Ishikawa, E. Ohtsuka and S. Nishimura. 1991. An endonuclease activity of *Escherichia coli* that specifically removes 8-hydroxyguanine residues from DNA. Mutat. Res. **254**:1-12.

Chung, M.H., H.S. Kim, E. Ohtsuka, H. Kasai, F. Yamamoto and S. Nishimura. 1991b. An endonuclease activity of human polymorphonuclear neutrophils that removes 8-hydroxyguanine residues from DNA, Biochem. Biophys. Res. Commun. **178**:1472-1478.

Clarke, E.A. 1984. Evaluation of the molecular design approach to the development of new dyes. Drug Metab. Rev. **15**: 997-1009.

Claycamp H.G. 1992. Phenol sensitization of DNA to subsequent oxidative damage in 8-hydroxyguanine assays. Carcinogenesis **13**:1289-1292.

Clayson D.B. 1987. The need for biological risk assessment in reaching decisions about carcinogenesis. Mutat. Res. **185**:243-269.

Clayson D.B., R. Mehta and F. Iverson. 1994. Oxidative DNA damage - The effects of certain genotoxic and operationally non-genotoxic carcinogens. Mutat. Res. **317**:25-42.

Coles, B. and B. Ketterer. 1990. The role of glutathione and glutathione transferase in chemical carcinogenesis. Crit. Rev. Biochem. Mol. Biol. **25**:47-70.

Combes, R.D. and R.B. Haveland-Smith. 1982. A review of the genotoxicity of food, drug and cosmetic colours and other azo, triphenylmethane and xanthene dyes. Mutat. Res. **98**: 101.

Conning, D.M. and J.C. Phillips. 1986. Comparative metabolism of BHA, BHT and other phenolic antioxidants and its toxicological relevance. Food Chem. Toxicol. **24**:1145-1148.

Corongiu, F.P., G. Poli, M.U. Dianzani, K.H. Cheeseman, T.F. Slater. 1986. Lipid peroxidation and molecular damage to polyunsaturated fatty acids in rat liver. Recognition of two classes of hydroperoxides formed under conditions *in vivo*. Chem. Biol. Interact. **59**:147-155.

Cotton, R.G.H. 1993. Current methods in mutation detection. Mutat. Res. **285**:124-144.

Cunningham, R.P., S.M. Saporito, S.G. Spitzer and B. Weiss. 1986. Endonuclease III (*nfo*) mutant of *Escherichia coli.* Proc. Natl. Acad. Sci. USA **82:**474-478.

Cunningham, R.P., S.M. Saporito, S.G. Spitzer and B. Weiss. 1986. Endonuclease IV (*nfo*) mutant of *Escherichia coli.* J. Bacteriol. **168**:1120-1127.

Cunningham, R.P. and B. Weiss. 1985. Endonuclease III(*nth*) mutants of *Escherichia coli* Proc. Natl. Acad. Sci. USA **82**:474-478.

Czeczot H., B. Tudek, B. Lambert, J. Laval and S. Boiteux. 1991. *Escherichia coli* Fpg protein and UvrABC endonuclease repair DNA damage induced by methylene blue plus visible light in vivo and in vitro. J. Bacteriol. **173**:3419-3424.

Daniel, J. 1962. The excretion and metabolism of edible food colours. Toxicol. Appl. Pharmacol. **4**:392-398.

Dasgupta C., A.M. Wu, R. Kahn, R.P. Cunningham and C.M. Radding. 1981. Concerted strand exchange and formation of Holliday strucutres by *Escherichia coli* RecA protein. Cell **25**:507-516.

David, M., G. Horvath, I. Schimke, M.M. Mueller and I. Nagy. 1993. Effects of the antioxidant butylated hydroxyanisole on cytosolic free calcium concentration. Toxicology **77**:115-121.

De Flora, S., C. Bennicelli, P. Zanacchi, F. D'Agostini and A. Camoirano. 1989. Mutagenicity of active oxygen species in bacteria and its enzymatic and chemical inhibition. Mutat. Res. **214**:152-158.

Delehanty, J., R.L. White and M.L. Mendelsohn. 1986. Approaches to determining mutation rates in human DNA. Mutat. Res. **167**:149-169.

DeMarini, D.M, J. Lewtas and H.E. Brockman. 1989. Utility of short-term tests for genetic toxicity. Cell Biol. Toxicol. **5**:189-199.

Demple, B., Y. Daikh, J. Greenberg and A. Johnson. 1986. Alkyation and oxidative damages to DNA: constitutive and inducible repair systems. Basic Life Sci. **39**:205-217.

Demple, B., A. Johnson and D. Fung. 1986. Exonuclease III and endonuclease IV removes 3' bloacks from DNA synthesis primers in hydrogen peroxide damaged *Escherichia coli* Proc. Natl. Acad. Sci. USA **83**:7731-7735.

Demple, B. and S. Linn. 1980. DNA N-glycosylases and UV repair Nature **287**:203-208

Dillon, D., R. Combes and E. Zeiger. 1994. Activation by caecal reduction of the azo dye D & C Red No. 9 to a bacterial mutagen. Mutagenesis **9**:295-299.

Dixon, W.T. and R.O.C. Norman. 1962. Free radicals formed during the oxidation and reduction of peroxides. Nature **196**: 891-892

Dizdaroglu, M. 1985. Formation of 8-hydroxyguanine moiety in deoxyribonucleic acid on gamma-irradiation in aqueous solution. Biochem. **24**:4476-4481.

Dizdaroglu, M. 1991. Chemical determination of free radical-induced damage to DNA. Free Rad. Biol. Med. **10**:225-242.

Dizdaroglu M., O.I. Aruoma and B. Halliwell. 1990. Modification of bases in DNA by copper ion-1, 10-phenanthroline complexes. Biochem. **29**:8447-8451.

Douglas, G.R, D.H. Blakey and D.B. Clayson. 1988. Genotoxicity tests as predictors of carcinogens: An analysis. Mutat. Res. **196**:83-93.

Dubin, P. and K.L Wright. 1975. Reduction of azo food dyes in cultures of *Proteus vulgaris.* Xenobiotica **5**:563-571.

Dzarlieva-Petrusevska, R.T. and N.E. Fusenig. 1985. Tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced chromosome aberrations in mouse keratinocyte cell lines: a possible genetic mechanism of tumor promotion. Carcinogenesis **6**:1447-1456.

Edwards, C.N. and R.D. Combes. 1981. An evaluation of the Drosphila zeste somatic eye mutation test for the detection of genotoxic azo dyes and an aromatic amine. Mutat. Res. **84**:221-226.

Edwards, C.N. and R.D. Combes. 1984. Mutagenicity studies of urine and faecal samples from rats treated orally with the food colourings brown FK and red 2Gl. **22**:593-597.

Elson, L.A., and F.W Warren. 1944.The metabolism of azo compounds. I. Azobenzene. Biochem. J. **38**:217-220.

Epe B., J. Hegler, D. Wild. 1988. Singlet oxygen as an ultimately reactive species in *Salmonella typhimurium* DNA damage induced by methylene blue/visible light. Carcinogenesis **10**:2019-2024.

Esancy, J.F., H.S. Freeman and L.D. Claxton. 1990. The effect of alkoxy substituents on the mutagenicity of some aminoazobenzene dyes and their reductive cleavage products. Mutat. Res. **238**:1-22.

Evans H.J. 1986. The role of human cytogenetics in studies of mutagenesis and carcinogenesis, in C. Ramel (Ed.), Genetic Toxicology of Environmental Chemicals, Part A: Basic Principles and Mechanisms of Action, Liss, New York, pp. 41-69.

Faila, E.S., C.C. Conaway and J.E. Mathis. 1989. Oxidative DNA and RNA damage in the livers of Sprague-Dawley rats treated with the hepatocarcinogen 2-nitropropane. Cancer Res. **49**:5518-5522.

Farr, S.B., R. D'Ari and D. Touati. 1986. Oxygen dependent mutagenesis in *Escherichia coli* lacking superoxide dismutase. Proc. Natl. Acad. Sci. USA **83**:8268.

Farr, S.B. and T. Kogama. 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium* Microbiol. Rev. **55**:561-585.

Farr, S.B., D. Touati and T. Kogoma. 1988. Effects of oxygen stress on membrane functionsin *Escherichia coli*. J. Bacteriol. **170**:1837-1842.

Faux S.P, J.E. Francis, A.G. Smith and J.K.Chipman. 1992. Induction of 8-hydroxydeoxyguanosine in *Ah*-responsive mouse liver by iron and Aroclor 1254. Carcinogenesis **13:**247-250.

Faux S.P, M. Gao, K. Chipman and L.S. Levy. 1992. Production of 8-hydroxydeoxyguanosine in isolated DNA by chromium(VI) and chromium(V). Carcinogenesis **13**:1667-1669.

Fiala, E.S., C.C. Conway and J.E. Mathis. 1989. Oxidative DNA and RNA damage in the livers of Sprague-Dawley rats treated with the hepatocarcinogen 2-nitropropane. Cancer Res. **49**:5518-5522.

Flohe, L. and F. Otting. 1984. Methods in Enzymology **105**:93-104.

Floyd, R.A. 1990. The role of 8-hydroxyguanine in carcinogenesis. Carcinogenesis **11**:1447-1550.

Floyd, R.A., J.J. Watson, P.K. Wong, D.H. Altmiller and R.C. Rickard. 1986. Hydroxyl free radical adduct of deoxyguanosine: sensitive detection and mechanisms of fomation. Free Rad. Res. Comms. **1**:163-172.

Floyd, R.A., M.S. West, K.L.Eneff and J.E. Schneider. 1990. Mediation of 8-hydroxy-guanine formation in DNA by thiazin dyes plus light. Free Radical Biol. Med. **8**:327-330.

Fore, H. and R. Walker. 1967. Studies of brown FK.I. Composition and synthesis of components. Food Cosmet. Toxicol. **5**:1-9.

Fore, H. and R. Walker. 1967. Studies of brown FK.II. Degradative changes undergone *in vitro* and *in vivo*. Food Cosmet. Toxicol. **5**:459-473.

Forster, R., M.H.L. Green and A. Priestley. 1980. Optimal levels of S9 in the Ames and fluctuation test: apparent importance of diffusion of metabolites from top agar. Carcinogenesis **1**:337-346.

Forsythe, S.J. 1990. The rapid detection of direct-acting DNA mutagens by electrical impedence with DNA repair deficient strains of *Escherichia coli*. Letts. Appl. Microbiol. **11**:30-32.

Foster, P.L. 1990. *Escherichia coli* strains with multiple DNA repair defects are hyperinduced for the SOS response. J. Bacteriol. **172**:4719-4720.

Fouts, J.R., J.J. Kamm and B.B. Brodie. 1957. Enzymatic reduction of prontosil and other azo dyes. J. Pharmacol. Exp. Ther. **120**:291-300.

Fraga, C.C., M.K. Shigenaga, J.W. Park, P. Degan and B.N. Ames. 1990. Oxidative damage to DNA during aging:8-hydroxy-2-deoxyguanosine in rat organ DNA and urine. Proc. Natl. Acad. Sci. USA **86**:6377-6381

Frankel, E.N. 1987. Secondary products of lipid oxidation. Chem. Phys. Lipids **44**:73-85.

Freeman, B.A. and J.D. Crapo. 1981. Biology of disease. Free radicals and tissue injury. Lab. Invest. **47**:412-426.

Freeman, H.S., Z. Hao, S.A.Mcintosh and K.P.Mills. 1988. Purification of some water soluble azo dyes by high-speed countercurrent chromatography. J. liquid Chrom. **11**:251-266.

Freeman, H.S., C.S. Williard and W.N.Hsu. 1986. Purification procedures for synthetic dyes: Part 1-Dry column chromatography. Dyes and Pigments. **7**:397-405.

Frenkel, K., Z. Zhong, H. Wei, J. Karkoszka, U. Patel, K. Rashid, M. Georgescu and J.J. Solomon. 1991. Quantitative high-performance liquid chromatography analysis and DNA oxidised *in vitro* and *in vivo*. Anal. Biochem. **169**:126-136.

Fridovich I. 1986a. Biological effects of the superoxide radical. Arch. Biochem Biophys. **247**:1.

Fridovich I. 1986b. Superoxide dismutases. Meth. Enzymol. **58**:61.

Fujita, S., Y. Okada and J. Peisach. 1981. Inhibition of azo reductase activity by antibodies against cytochromes P450 and P448. Biochem. Biophys. Res. Commum. **102**:492-498.

Fujita, S. and J. Peisach. 1978. Liver microsomal cytochromes P450 and azoreductase activity. J. Biol. Chem. **253**:4512-4513.

Fujita, S. and J. Peisach. 1982. The Stimulation of microsomal azoreduction flavins. Biochem Biophys. Acta. **719**:178-189.

Fusening , N.E., Dzarlieva-Petrusevska, R.T. and D. Breitkreutz. 1985. Phenotypic and cytogenetic characteristics of different stages during spontaneous transformation of mouse keratinocytes *in vitro* Carcino. Compr. Surv. **9**:293-326.

Garner, R.C., C.N. Martin and D.B. Clayson. 1984. Carcinogenic aromatic amines and related compounds in: C.E. Searle (ed.) Chemical Carcinogenesis, Vol 1, American Chemical Society, Washington, DC, 1984, p. 175

Garner, R.C. and C.A. Nutman. 1977. Testing of some azo dyes and their reduction products for mutagenicity using *Salmonella typhimurium* TA1538. Mutat. Res. **44**:9-19.

Gasparic, J. 1977. Chemical degradation methods. pp. 299-316 *In*: The Analytical Chemistry of Synthetic Dyes (K. Venkataraman. Ed.). John Wiley and Sons, New York.

Gee, P., R.H.C. San, A.J. Davison and H.F. Stich. 1992. Clastogenic and mutagenic actions of active species generated in the 6-hydroxydopamine/oxygen reaction: effects of scavengers of active oxygen, iron, and metal chelating agents. Free Rad. Res. Comms. **16**:1-10.

Gingell, R. and R. Walker. 1971. Mechanism of azo reduction by *Streptococcus faecalis* II. The role of soluble flavins. Xenobiotica **1**:231-239.

Goerlich, O., P. Quillardet and M. Hofnung. 1989. Induction of the SOS response by hydrogen peroxide in various *Escherichia coli* mutants with altered protection against oxidative DNA damage. J. Bacteriol. **171**:6141-6147.

Goldin, B.R. and S.L. Gorbach. 1976. The relationship between diet and rat faecal bacterial enzymes implicated in colon cancer. J. Natl. Cancer Inst. **57**:371.

Goszczynski S., A. Paszczynski, M.B.Pasti-Grigsby, R.L. Crawford and D.L. Crawford. 1994. New pathway for degradation of sulphonated azo dyes by microbial peroxidases of *Phanerochaete chrysosporium* and *Streptomyces chromofuscus.* J. Bacteriol. **176**:1339-1347.

Gower, J.D. 1988. A role for dietary lipids and antioxidants in the activation of carcinogens. Free Radical Biol. Med. **5**:95-11.

Graves, H.M and L.G. Johnston. 1988. The effect of metallization on singlet oxygen formation by azo dyes. J. Photochem. Photobiol., A:Chemistry **43**:183-192

Green, M.H.L and W.J. Muriel. 1976. Mutagen testing using trp^+ reversion in *Escherichia coli* Mutat. Res. **38**:3-32.

Greenberg, J.T., P. Monach, J.H. Chou, P.d. Josephy and B. Demple. 1990. Positive control of a global antioxidant defence regulon activated by superoxide-generating agents in *Escherichia coli* Proc. Natl. Acad. Sci. USA. **87**:6181-6185.

Gregory, P. 1986. Azo dyes: structure - carcinogenicity relationships. Dyes and Pigments **7**:45-56.

Greim, H., W. Goggelmann, K.H. Summer and T. Wolff. 1980. Mutagenicity testing with *Salmonella* microsome test. Arch. Toxicol. **46**:31-40.

Grollman, A.P. 1992. Structural basis for the mutagenic specificity of 8-oxoguanine-DNA, in: R. Sarma (Ed.), Proc. Seventh Conversation in Biomolecular Stereodynamics, Adenine Press, New York.

Grossman, L. and A.T. Yeung. 1990. The UvrABC endonuclease system of *Escherichia coli* - a view from Baltimore. Mutat. Res. **236**:213-221.

Gupta, R.C. 1987. 32P-post-labeling assay to measure carcinogen-DNA adducts. Prog. Exp. Tumor Res. **31**:21-32.

Gupta, R.C., S.K. Goel, K. Earley, B. Singh and J.K. Reddy. 1985. 32P-post labeling analysis of peroxisome proliferator-DNA adduct formation in rat liver *in vivo* and hepatocytes *in vitro*. Carcinogenesis **6**:933-936.

Gutteridge, J.M.C., L. Maidt and L. Poyer. 1990. Superoxide dismutase and Fenton chemistry. Biochem J. **269**:169-174.

Gutteridge, J.M.C., Richmond R. and B. Halliwell. 1979. Inhibition of the iron-catalysed formation of hydroxyl radicals from superoxide and of lipid peroxidation by desferrioxamine. Biochem. J. **184**:469-472.

Haley, T.J. 1975. Benzidine revisited: a review of the literature and problems associated with the use of benzidine and its congeners. Clin. Toxicol. **8**:13.

Halliwell, B. 1987. Oxidative damage, lipid peroxidation and antioxidant protection in chloroplasts. Chem. Phys. Lipids **44**:327.

Halliwell, B. and O.I. Aruoma. 1991. DNA damage by oxygen-derived species: its mechanism and measurement in mammalian systems. FEBS lett. **281**:9-19.

Halliwell, B. and M. Dizdaroglu. 1992. The measurement of oxidative damage to DNA by HPLC and GC/MS techniques. Free Rad. Res. Comms. **16**:75-87.

Halliwell, B. and J.M.C. Gutteridge. 1984. Lipid peroxidation, oxygen radicals, cell damage and antioxidant therapy. Lancet **i**:1396.

Halliwell, B. and J.M.C. Gutteridge. 1985. Oxygen radicals and the nervous system. Trends Neurosci. **8**:22.

Halliwell, B. and J.M.C Gutteridge. 1986. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. Arch. Biochem. Biophys. **246**:501.

Halliwell, B. and J.M.C Gutteridge. 1990. Role of free radicals and catalytic metal ions in human disease: An overview. Methods Enzymol. **186**:1-38.

Halliwell, B. and J.M.C. Gutteridge. 1990. The antioxidants of human extracellular fluids. Arch. Biochem. Biophys. **280**:1-8.

Halliwell, B. , M. Wasil and M. Grootveld. 1987. Biologically significant scavenging of the myeloperoxidase-derived oxidant hypochlorous acid by ascorbic acid. FEBS Lett. **213**:15

Han, J.-H. 1992. Effects of various chemical compounds on spontaneous and hydrogen peroxide-induced in strain TA104 of *Salmonella typhimurium.* Mutat. Res. **266**:77-84.

Hanawalt, P.C., P.K. Copper, A.K. Ganeson and C.A. Smith. 1979. DNA repair in bacteria and mammalian cells. Ann Rev. Biochem. **48**:783-836.

Hariharan, P.V. and P.A. Ceruth. 1977. Formation and repair of x-ray induced thymine damage in *Micrococcus radiodurans* J. Mol. Biol. **66**:65-81.

Harris, G., S. Bashir and P.G. Winyard. 1994. 7,8-Dihydro-8-oxo-2'-deoxyguanosine present in DNA is not simply an artefact of isolation. Carcinogenesis **15**:411-413.

Hartman, C.P., A.W. Andrews and K-T. Chung. 1979. Production of a mutagen from ponceau 3R by a human intestinal anaerobe. Infect. Immun. **23**:686-689.

Hartman C.P, G. E. Fulk and A.W. Andrews. 1978. Azo reduction of trypan blue to a known carcinogen by a cell-free extract of a human intestinal anaerobe. Mutat Res. **58**:125-132.

Hartman, P.E. 1990. Ergothioneine as antioxidant. Methods Enzymol. **186**:310-318.

Haug, M., A. Schmidt, B. Nortemann, D.C. Hempel, A. Stolz and H.J. Knackmuss. 1991. Mineralisation of the sulphonated azo dye mordant yellow 3 by a 6-aminonaphthalene-2-sulfonate-degrading bacterial consortium. App. Environ. Microbiol. **57**:3144-3149.

Haveland-Smith, R.B. and R.D. Combes. 1980. Screening of food dyes for genotoxic activity. Fd. Cosmet. Toxicol. **18**:215-221.

Haveland-Smith, R.B. and R.D. Combes. 1980. Genotoxicity of the food colours red 2G and brown FK in bacterial systems: use of structurally-related dyes and azo-reduction. Fd. Cosmet. Toxicol. **18**:223-228.

Haveland-Smith, R.B. and R.D. Combes. 1982. Studies on the genotoxicity of the food colour brown FK and its component dyes using bacterial assays. Mutat. Res. **105**:51-58.

Hayashi, Y. 1992. Overview of genotoxic carcinogens and non-genotoxic carcinogens. Exp. Toxic. Pathol. **44**:465-472.

Hayatsu, H., S. Arimoto and T. Negishi. 1988. Dietary inhibitors of mutagenesis and carcinogenesis. Mutat. Res. **202**:429-446.

Heiss, G.S., B. Gowan and E.R. Dabbs. 1992. Cloning of DNA from a *Rhodococcus* strain conferring the ability to decolorize sulfonated azo dyes. FEMS Microbiol. Letts. **99**:221-226.

Henschler, D. and D. Wild. 1985. Mutagenic actvity in rat urine after feeding with the azo dye tartrazine. Arch. Toxicol. **57**:214-215.

Hernandez, P.H., P. Mazel, and J.R. Gillette. 1967. Studies on the mechanism of action of mammalian hepatic azoreductases. I. Azoreductase activity of reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase. Biochem. Pharmacol. **16**:1859-1875.

Hill-Perkins, M., M.D. Jones and P. Karran. 1986. Site specific mutagenesis *in vivo* by single methylated or deaminated purine bases. Mutat. Res. **162**:153-163

Hinrichsen, L.I., R.A. Floyd and O.Sudilovsky. 1990. Is 8-hydroxydeoxyguanosine a mediator of carcinogenesis by a choline-devoid diet in the rat liver? Carcinogenesis **11**:1879-1881.

Hix, C., L. Oglesby, P. MacNair, M. Sieg and R. Langenbach. 1983. Bovine bladder and liver cell and homogenate-mediated mutagenesis of *Salmonella typhimurium* with aromatic amines. Carcinogenesis **4**:1401-1407.

Hoffman, M.E., A.C. Mello-Filho and R. Meneghini. 1984. Correlation between cytotoxic effect of hydrogen peroxide and the yield of DNA strand breaks in cells of different species. Biochim. Biophys. Acta. **781**:234-238.

Honohan, T., F.E. Enderlin, B.A. Ryerson and T.M. Parkinson. 1977. Intestinal absorption of polymeric derivatives of the food dyes sunset yellow and tartrazine in rats. Xenobiotica **7**:765.

Horitsu, H., M. Takada, E. Idaka, M. Tomoyeda and T. Ogawa. 1977. Degradation of p-aminoazobenzene by *Bacillus subtilis.* Eur. J. Appl. Micorbiol. **4**:217-224.

Huang M-T., G.T. Miwa and A.Y.H. Lu. 1979. Rat liver cytosolic azoreductase. effects. J. Biol. Chem. **254**:3930-3934.

Hutchinson, F. 1985. Chemical changes induced in DNA by ionising radiation. Prog. Nucleic Acid Res. Mol. Biol. **32**:115-154.

Hyman, J., Leifer, Z. and H.S. Rosenkranz. 1980. The *Escherichia coli* $PolA_1$- assay, a quantitative procedure for diffusible and non-diffusible chemicals. Mutat. Res. **74**:107-111.

Idaka, E., H. Horitsu and T. Ogawa. 1987. Some properties of azoreductase produced by *Pseudomonas cepacia.* Bull. Environ. Contam. Toxicol. **39**:982-989.

Imlay, J.A., S.M. Chin and S. Linn. 1988. Toxic DNA Damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. Science **240**:640-642.

Imlay, J.A. and S. Linn. 1987. Mutagenesis and stress responses induced in *Escherichia coli* by hydrogen peroxide. J. Bacteriol. **169**:2967-2976.

Imlay, J.A. and S. Linn. 1988. DNA damage and oxygen radical toxicity. Science **240**: 1302-1304.

Inoue, S., K. Ito, K. Yamamoto and S. Kawanishi. 1978. Caffeic acid causes metal-dependent damage to cellular and isolated DNA through hydrogen peroxide formation. Carcinogenesis **13**:1497-1502.

Iversen, O.H. and E.G. Astrup. 1984. The paradigm of two-stage carcinogenesis: a critical attitude. Cancer Invest. **2**:51-60.

Jacobson F.S., R.W. Morgan, M.F. Christman and B.N. Ames. 1989. An alkyl hydroperoxide reductase from *Salmonella typhimurium* involved in the defence of DNA against oxidative damage. J. Biol. Chem. **264**:1488-1496.

Jeffrey, A. M., K.W. Jennette, S.H. Blobstein, I.B. Weinstein, F.A. Beland, R.G. Harvey, H. Kasai, I. Miura and K. Nakanishi. 1976. Structure of 7,12-dimethylbenz[a]anthracene-guanosine adducts. Proc. Natl. Acad. Sci. USA **73**:2311-2315.

Jinqi, L. and L. Houtian. 1992. Degradation of azo dyes by algae. Environ. Pollution **75**:273-278.

Joachim, F., A. Burrell and J. Anderson. 1985. Mutagenicity of azo dyes in the Salmonella/microsome assay using *in vitro* and *in vivo* activation. Mutat. Res. **156**:131-138.

Jonas S.J., P.A. Riley and R.L. Willson. 1989. Hydrogen peroxide cytotoxicity. Biochem J. **264**:651-655.

Kada, T., K. Hirano and Y.Shirasu. 1980. Screening of environmental chemical mutagens by the *rec*-assay system with *Bacillus substilis*, in Chemical Mutagens:principle and methods for their detection. de Serres, F.J. and A. Hollaender (eds.) Chapter 6, pp 149-173.

Kadlubar, F.F., J.A. Miller and E.C. Miller. 1976. Microsomal N-oxidation of the hepatocarcinogen N-methyl-4-aminoazobenzene and the reactivitiy of N-hydroxy-N-methyl-4-aminoazobenzene. Cancer Res. **36:**1196-1206.

Kasai, H., P.F. Crain, Y. Kuchino, S. Nishimura, A. Ootsuyama and H. Tanooka. 1986. Formation of 8-hydroxyguanine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair. Carcinogenesis **7**:1849-1851.

Kasai, H., A. Iida, Z. Yamaizumi, S. Nishimura and H. Tanooka. 1990. 5-Formyldeoxyuridine: a new type of DNA damage induced by ionizing radiation and its mutagenicity to Salmonella strain TA102. Mutat. Res. **243**:249-253.

Kasai, H. and S. Nishimura. 1984. Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. Nucl. Acids Res. **12**:2137-2145.

Kasai, H., S. Nishimura and Y. Kurokawa. 1987. Oral administration of the renal carcinogen, potassium bromate, specifically produces 8-hydroxydeoxyguanosine in rat target organs. Carcinogenesis **8**:1959-1961.

Kato, T. and Y. Shinoura. 1977. Isolation and characterisation of mutants of *Escherichia coli* deficient in induction of mutants by ultraviolet light. Mol. Gen. Genet. **156**:121-131.

Kimura, T., M. Kodama and C. Nagata. 1979. Nitroxide radicals generated from carcinogenic aminoazo dyes during their metabolism *in vivo* and in enzymatic system *in vitro.* Biochem. Pharmacol. **28**:557-560.

Kimura, T., M. Kodama and C. Nagata. 1982. N-hydroxylation enzymes of carcinogenic aminoazo dyes: Possible involvement of cytochrome P-448. Gann **73**:55-62.

King, M.M. and P.B. McCay. 1983. Modulation of tumor incidence and possible mechanisms of inhibition of mammary cacinogenesis by dietary antioxidants. Cancer Res. **42**:2485s-2490s.

Klopman, G. 1984. Artificial intelligence approach to structure-activity studies. Computer automated structure evaluation of biological activity of organic molecules. J. Am. Chem. Soc. **106**:7315-7321.

Klopman, G., M.R. Frierson and H.S. Rosenkranz. 1990. The structural basis of the mutagenicity of chemicals in *Salmonella typhimurium*: The Gene-Tox Data Base. Mutat. Res. **22**:1-50.

Kohda, K., M. Tada, H. Kasai, H. Inoue, S. Iwai, K. Miura, E. Ohisuka and S. Nishimura. 1986. Formation of 8-hydroxyguanine in cellular DNA exposed to the carcinogen 4-nitroquinoline 1-oxide. Biochim. Biophys. Res. Commun. **139**:626-632.

Koppenol, W.H. 1985. The reaction of ferrous EDTA with hydrogen peroxide: evidence against hydroxyl radical formatio. J. Free Radicals Biol. Med. **1**:281-285.

Kow, Y.W., S.S. Wallace and B.V. Houten. 1990. UvrABC nuclease complex repairs thymine glycol, an oxidative DNA base damage. Mutat. Res. **235**:147-156

Kulla, H.G. 1983. Interference of aromatic sulpho groups in the microbial degradation of azo dyes orange I and orange II. Arch. Microbiol. **135**:1-7.

Kuroki, T., C. Malaveille, C. Drevon, C. Piccoli, M. Macleod and J.K. Selkirk. 1979. Critical importance of microsome concentration in mutagenesis assay with V79 Chinese hamster cells, Mutation Research, **63**:259-272.

Lagorio, S., C. Tagesson, F. Forastiere, I. Iavarone, O. Axelson and A. Carere. 1994. Exposure to benzene and urinary concentrations of 8-hydroxydeoxyguanosine, a biological marker of oxidative damage to DNA. Occup. Environ. Med. **51**:739-743.

Larsen, L.C., T. Meyer and R.R. Scheline. 1976. Reduction of sulphonated water-soluble azo dyes by caecal microorganisms from the rat. Acta. Pharmacol. Toxicol. **38**:353.

Laughton, M.J., B. Halliwell, P.J. Evans and J.R.S. Hoult. 1989. Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin. Biochem. Pharmacol. **38**:2859-2865.

Lawley, P.D. 1989. Mutagens as carcinogens: development of current concepts. Mutat. Res. **213**:3-25.

Learoyd, S.A., R.G.Kroll and C.F. Thurston. 1992. An investigation of dye reduction by food-borne bacteria. J. Appl. Bacteriol. 72:479-485.

Lebedev, A.V., D.O. Levitsky and V.A. Loginov. 1992. Oxygen as an indicator of divalent cation permeability through biological and model lipid membranes. Adv. Myocardial. **3**:425-438.

Leifer, Z., T. Kada, M. Mandel, E. Zeiger, C. Stafford and H.S. Rosenkranz. 1981. An evaluation of tests using DNA repair-deficient bacteria for predicting genotoxicity and carcinogenicity. A report of the US. EPA's GeneTox Program, Mutat. Res. **87**:211-297.

Lesko, S.A., R.J. Lorentzen and P.O.P.Ts'o. 1980. Role of superoxide in DNA strand scission. Biochem. **19**:3023-3028.

Levin, D.E., M. Hollstein, M. Christman, E. Schwiers and B.N. Ames. 1982. A new *Salmonella* tester strain (TA102) with A.T base pairs at the site of mutation detects oxidative mutagens. Proc. Natl. Acad. Sci. USA. **79**:7445-7449.

Levin, J.D., A.W. Johnson and B. Demple. 1988. Homogenous *Escherichia coli* endonuclease IV. Characterisation of an enzyme that recognises oxidative damage in DNA. J. Biol. Chem. **263**:8066-8071.

Levin , J.D., R. Shapiro and B. Demple. 1991. Metalloenzymes in DNA repair. *Escherichia coli* endonuclease IV and *Saccharomyces cerevisiae* Apn1. J. Biol. Chem. **266**:22893-22898.

Levine, W.G. 1991. Metabolism of azo dyes: implication for detoxification and activation. Drug. Metab. Rev. **23**:253-309.

Levine, W.G. and H. Raza. 1988. Mechanism of azoreduction of dimethylaminoazobenzene by rat liver NADPH-cytochrome P-450 reductase ans partially purified cytochrome P-450: oxygen and carbon monoxide sensitivity and stimulation by FAD and FMN. Drug Metab. Dispos. **16**:441-448.

Lijinsky W. 1993. Species differences in carcinogenesis. In Vivo. **7**:65-72.

Lin, G.H.Y. and W.E. Solodar. 1988. Structure-activity relationship studies on the mutagenicity of some azo dyes in the salmonella/microsome assay. Mutagenesis **3**:311-315.

Lin, J.J. and A. Sancar. 1989. A new mechanism for repairing oxidative damages to DNA: (A)BC excinuclease removes AP sites and thymine glycols from DNA. Biochem. **28**:7979-7984.

Lindahl, T. 1982. DNA repair enzymes. Annu. Rev. Biochem. **51**:61-87.

Little, J.W. and D.W. Mount. 1992. The SOS regulation system of *Escherichia coli.* Cell **29**:11-22.

Liu, Z.P. and H.F. Yang. 1989. The decolourisation and biodegrading metabolism of azo dyes by *Pseudomonas* S-42. Wei. Sheng. Wu. Hsueh. Pao. **29**:418-426.

Ljungquist, S. 1977. A new endonuclease from *Escherichia coli* acting at apurinic sites in DNA/ J. Biol. Chem. **252**:2808-2814.

Loeb L.A., T.A. Kunkle. 1982. Fidelity of DNA synthesis. Annu. Rev. Biochem. **52**:429-457.

Loewen, P.C. 1984. Isolation of catalase deficient *Escherichia coli* mutants and genetic mapping of *katE*, a locus that affects catalase activity. J. Bacteriol. **157**:622-626.

Lunn, C.A. and V.P. Pigiet. 1987. The effect of thioredoxin on the radiosenstivity of bacteria. Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. **51**:29-38.

Lutz, W.K. and P. Maier. 1988. Genotoxic and epigenetic chemical carcinogenesis: one process, different mechanisms. Trends Pahrmacol. Sci. **9**:322-326.

Machlin, L.J. 1984. handbook of vitamins, Marcel Dekker Inc., New York and Basel.

Mackey B.M., and D.A. Seymour. 1987. The effect of catalase on recovery of heat-injured DNA-repair mutants of *Escherichia coli.* J. Gen. Micro. **133**:1601-1610.

Mallett, A.K., L.J. King and R. Walker. 1985. Solubilisation, purification and reconstitution of hepatic microsomal azoreductase activity. Biochem. Pharmacol. **34**:337-342.

Manning, B.W, C.E. Cerniglia and T.W.Federle. 1985. Metabolism of the benzidine-based azo dye direct black 38 by human intestinal microbiota. Appl. Environ. Microbiol. **50:**10-15.

Marletta, M.A. 1991. Nitric oxide, nitrovasodilators, and L-arginine - an unusual relationship. West J. Med. **154**:107-109.

Marnett, L.J. 1987. Peroxyl free radicals: Potential mediators of tumor initiation and promotion. Carcinogenesis. **8**:81265-81373.

Maron, D.M and B.N. Ames. 1983. Revised methods for the salmonella mutagenicity test. Mutat. Res. **113**:173-215.

Martin, C.N. and J.C. Kennelly. 1981. Rat liver microsomal azo-reductase activity on four azo dyes derived form benzidine,3,3'-dimethylbenzidine or 3,3'-dimethoxybenzidine. Carcinogenesis **2**:307.

Mashino, T. and I. Fridovich. 1987. Superoxide radical initiates the autoxidation of dihydroxyacetone. Arch. Biochem. Biophys. **254**:547-551.

Mason R.P., F.J. Peterson and J.L. Holtzman. 1978. The role of the azoanion free radical metabolite in the reduction of oxygen to superoxide. Molecular Pharm. **14**:665-671.

McBride, T.J., B.D. Preston and L.A. Loeb. 1991. Mutagenic spectrum resulting from DNA damage by oxygen radicals. Biochem. **30**:207-213.

McBride, T.J., J.E. Schneider, R.A. Floyd and L.A. Loeb. 1992. Mutations induced by methylene blue plus light in single stranded M13mp2, Proc. Natl. Acad. Sci. USA. (In press)

McCann, J. and B.N. Ames. 1976. Detection of carcinogens as mutagens in the Salmonella/ microsome test: Assay of 300 chemicals: Discussion. Proc. Natl. acad. Sci. 73:950.

McCann, J. and B.N. Ames. 1977. The Salmonella/microsome mutagenicity test: predictive value for animal carcinogenicity. In: Hiatt, H.H., J.D. Watson and J.A. Winsten (Eds.) Origins of human cancer. Cold Spring Habor Laboratory, New York, p1431.

McCann, J., E. Choi, E. Yamasaki and B.N. Ames. 1975. Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA. **72**:5135-5139.

McCarroll, N.E., B.H. Keech and C.E. Piper. 1981a. A microsuspension adaption of the *Bacllus subtilis* 'rec' assay. Environ. Mutagen. **3**:607-616.

McCarroll, N.E., C.E. Piper and B.H. Keech. 1981b. An *Escherichia coli* microsuspension assay for the detection of DNA damage induced by direct acting and promutagens. Environ. Mutagen. **3**:607-616.

McCord, J.M. 1977. *In* Michelson, A.M., McCord, J.M and Fridovich, I. (eds), Superoxide and superoxide dismutase, Academic Press, London, p11-17.

McCord, J.M. and E.D. Day. 1978. Superoxide-dependent production of hydroxyl radical catalysed by iron-EDTA complex. FEBS Lett. **86**:139-142.

McCoy, E.C., W.T. Speck and H.S. Rosenkranz. 1977. Activation of a procarcinogen to a mutagen by cell-free extracts of anaerobic bacteria. Mutat. Res. **46**:261-264.

Mechsner, K. and K. Wuhrmann. 1982. Cell permeability as a rate limiting factor in the microbial reduction of sulphonated azo dyes. Euopean J. Appl. Microbiol. Biotechnol. **15**:123-126.

Meister, A. and M.E. Anderson. 1983. Glutathione. Annu. Rev. Biochem. **57**:711-760.

Mello-Filho, A.C., M.E. Hoffmann and R. Meneghini. 1984a. Cell killing and DNA damage by hydrogen peroxide are mediated by intracellular iron. Biochem. J. **218**:273.

Mello-Filho, A.C. and R. Meneghini. 1984b. *In vivo* formation of single-strand breaks in DNA by hydrogen peroxide is mediated by the Haber-Weiss reaction. Biochim. Biophys. Acta. **781**:56-63.

Mello-Filho, A.C. and R. Meneghini. 1985. Protection of mammalian cells by o-phenanthroline from lethal and DNA-damaging effects produced by active oxygen species. Biochim. Biophys. Acta. **847**:82.

Mello-Filho, A.C. and R. Meneghini. 1991. Iron is the intracellular metal involved in the production of DNA damage by oxygen radicals. Mutat. Res. **251**:109-113.

Michaels, M.L., C. Cruz, A.P. Grollman and J,H, Miller. 1992. Evidence that MutY and MutM combine to prevent mutations by an oxidatively damaged form of guanine in DNA. Proc. Natl. Acad. Sci. USA **89**:7022-7025.

Miller, E.C. 1978. Some current perspectives on chemical carcinogenesis in humans and experimental animals. Cancer Research. **38**:1479-1496 .

Miller, E.C. and J.A. Miller. 1976. Chapter 16: The metabolism of chemical carcinogens to reactive electrophiles and their possible mechanisms of action in carcinogenesis, in: C. E. Searle (Ed.) Chemical carcinogens, A.C.S. Monogr. No. **173**:737-762.

Miller, J.A. and E.C. Miller. 1948. The Carcinogenicity of certain derivatives of p-dimethylaminoazobenzene in the rat. J. Exp. Med. **87**:139-156.

Miller, J.A. and E.C. Miller. 1969. The metabolic activation of carcinogenic aromatic amines and amides. Progr. Exp. Tumour. **11**:273-301.

Miller, J.A. and E.C. Miller. 1977. Ultimate chemical carcinogenesis reactive mutagenic electrophiles. Cold Spring Harbor. Cell Proliferation **4**:605-627.

Minotti G. and S.D. Aust. 1987. The role of iron in the initiation of lipid peroxidation. Chem. Phys. Lipids **44**:191.

Mirsalis, J.C. 1987. *In vivo* measurement of unscheduled DNA synthesis and S-phase synthesis as an indicator of hepatocarcinogenesis in rodents. Cell Biol. Toxicol. **3**:165-173.

Mito, S., Q-M. Zhang and S. Yonei. 1993. Isolation and characterisation of *Escherichia coli* strains containing new gene fusions (*soi::lacZ)* inducible by superoxide radicals. J. Bacteriol. **175**:2645-2651.

Mock, M., M. Crasnier, E. Duflot, V. Dumay and A. Danchin. 1991. Structural and functional relationships between *Pasteurella multocida* and enterobacterial adenylate cyclases. J. Bacteriol. **173**:6265-6269.

Monsen T.J., S.E. Holm and L.G. Burman. 1983. A general method for cell lysis and preparation of deoxyribonucleic acid from streptococci. Microbiol. Letts. **16**:19-24.

Moriishi, K., B. Syuto, N. Yokosawa, K. Oguma and M. Saito. 1991. Purification and characterisation of ADP-ribosylytransferases (exoenzyme C3) of *Clostridium botulinum* type C and D strains. J. Bacteriol. **173**:6025-6029.

Morikawa, K., O. Matsumoto, M. Tsujimoto, K. Katayanagi, M. Ariyoshi, T. Dot, M. Ikehara, T. Inaoka and E. Ohtsuka. 1992. X-Ray structure of T4 endonuclease V: an excision repair enzyme specific for a pyrimidine dimer. Science. **256**:523-526

Moriya M. and A.P. Grollman. 1993. Mutations in the *mut*Y gene of *Escherichia coli* enhance the frequency of targeted G:C→T:A transversions induced by a single 8-oxoguanine residue in single-stranded DNA. Mol. Gen. Genet. **239**:72-76.

Moriya, M., C. Ou, V. Bodepudi, F. Johnson, M. Takeshita, A.P. Grollman. 1991. Site-specific mutagenesis using a gapped duplex vector: a study of translesion synthesis past 8-oxodeoxyguanosine in *Escherichia coli*. Mutat. Res. **245**:281-288.

Morton, K.C., C.M. King and K.P. Baetcke. 1979. Metabolism of benzidine to N-hydroxy-N,N'-diacetylbenzidine and subsequent nucleic acid binding and mutagenicity. Cancer Res. **39**:3107-3113.

Mosbaugh, D.W. and S. Linn. 1982. Characterisation of the action of *Escherichia coli* DNA polymerase I at incisions produced by repair endodeoxyribonucleases. J. Biol. Chem. **257**:575-583.

Mueller, G.C. and J.A. Miller. 1950. The reductive cleavage of 4-dimethylamino azobenzene by rat liver: Reactivation of carbon dioxide treated homogenates by riboflavin-adeninedinucleotide. J. Biol. Chem. **185**:145-154.

Muller J. and S. Janz. 1992. Assessment of oxidative DNA damage in the oxyR-deficient SOS chromotest strain *Escherichia coli* PQ300. Environ. Mol. Mutagen. **20**:297-306.

Murphy M.E. and H. Seis. 1991. Reversible conversion of nitroxyl anion to nitric oxide by superoxide dismutase. Proc. Natl. Acad. Sci. USA **88**:10860-10864.

Myrnes, B., K.E. Giercksky and H.Krokan. 1982. Repair of O6-methyl-guanine residues in DNA takes place by a similar mechanism in extracts from HeLa cells, human liver, and rat liver. J. Cell. Biochem. **20**:381-392.

Nabben, F.J., M.V. Lafleur, J.C. Sikkers, A.c. Loman, J. Retel and H. Loman. 1984. Repair damage in double stranded phi X174 (RF) DNA due to radiation induced water radicals. Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. **45**:379-388.

Nakayama, T., T. Kimura, M. Kodama and C. Nagata. 1983. Generation of hydrogen peroxide and superoxide anion from active metabolites of naphthalamines and aminoazo dyes: its possible role in carcinogenesis. Carcinogenesis **4**:765-769.

Nicolson, G.L. 1987. Tumor cell instability, diversification, and progression to the metastatic phenotype: from oncogenes to oncofetal expression. Cancer Res. **47**:1473-1487.

O'Connor, T.R., R.J. Graves, G. de Murcia, B. Castaing and J. Laval. 1993. Fpg protein of *Escherichia coli* is a zinc finger protein whose cysteine residues have a structural and/or functional role. J. Biol. Chem. **268**:9063-9070.

O'Connor, T.R. and J. Laval. 1989. Physical association of the 2,6-diamino-4-hydroxy-5N-formamidopyrimidine-DNA glycosylase of *Escherichia coli* and an activity nicking DNA at apurinic/apyrimidinic sites. Proc. Natl. Acad. Sci. USA **86**:5222-5226.

Oberley, L. W. and D.R. Spitz. 1984. Assay of superoxide dismutase activity in tumour tissue. *In*: Methods in Enzymology, Vol. 105 (L. Packer. Ed.). Academic Press, Inc London, pp457-464.

Orrenius, S., D.J. McConkey, G. Bellomo and P. Nicotera. 1989. Role of calcium in toxic cell killing. Trends Pharm. Sci. **10**:281-285.

Ortiz de Montellano, P.R. 1987. Control of the catalytic activity of prosthetic heme by the structure of hemoproteins. Acc. Chem. Res. **20**:289-294.

Packer, J.E., T.F. Slater and R.L. Wilson. 1979. Direct observation of a free radical interaction between vitamin E and vitamin C. Nature **278**:737-738.

Park, J-W. 1991. A correlation between oxygen radical-induced mutations and DNA adducts formation in *Salmonella typhimurium.* Korean Biochem J. **24**:387-393.

Parkin D.M., E. Laara and C.S. Muir. 1988. Estimates of the worldwide frequency of sixteen major cancers in 1980. Int. J. Cancer. **41**:184-197.

Pasti-Grigsby, M.B., A. Paszczynski, S. Goszczynski, D.L. Crawford and R.L. Crawford. 1992. Influence of aromatic substitution patterns on azo dye degradability by *Streptomyces* spp. and *Phanerochaete chrysosporium.* Appl. Environ. Mircobiol. **58**:3605-3613.

Paszczynski, A., M.B.Pasti-Grigsby, S. Goszczynski, R.L. Crawford and D.L. Crawford. 1992. Mineralisation of sulphonated azo dyes and sulfanilic acid by *phanerochaete chrysosporium* and *Streptomyces chromofuscus.* Appl. Environ. Microbiol. **58**:3598-3604.

Perly, B.I., C.P. Smith, L.Hughes, G.W. Burton and K.U. Ingold. 1985. Estimation of the location of natural α-tocoperol in lipid bilayers by C13-NMR spectroscopy. Biochim. biophys. Acta. **819**:131-135.

Peterson, F.J., J.L. Holtzman, D. Crankshaw and R.P. Mason. 1988. Two sites of azoreduction in the monooxygenase system. Mol. Pharmacol. **34**:597-603.

Pitot, H.C. 1989. Progression: the terminal stage in carcinogenesis. Jpn. J. Cancer Res. **80:**599-607.

Pitot, H.C. and Y.P. Dragon. 1991. Facts and theories concerning the mechanisms of carcinogenesis. FASEB J. **5**:2280-2286.

Poli, G., K. Cheeseman, T.F. Slater and M.U. Dianzani. 1981. The role of lipid peroxidation in carbon tetrachloride induced damage to liver microsomal enzymes: comparative studies *in vitro* using microsomes and isolated liver cells. Chem. Biol. Interact. **37**:13-24.

Poli, G., D. Cottalasso, M.A. Pronzato, E. Chiarpotto, F. Biasi, F.P. Corongiu, U.M. Marinari, G. Nanni, M.U. Dianzani. 1990. Lipid peroxidation and covalent binding in the early functional impairment of liver Golgi apparatus by carbon tetrachloride. Cell Biochem. Funct. **8**:1-10.

Pool, B.L. and D. Schmahl. 1987. What's new in mutagenicity and carcinogenicity - status of short-term assay systems in genetic toxicology and carcinogenesis. Pathol. Res. Pract. **182**:704-712.

Poulsen, E. 1991. Safety evaluation of substances consumed as technical ingredients (food additives). Food. Addit. Contam. **8**:125-133.

Poupko, J.M., T. Radomski, R.M. Santella and J.L. Radomski. 1983. Organ, species, and compound specificity in the metabolic activation of primary aromatic amines. J. Natl. Cancer. Inst. **70**:1077-1080.

Powell, R., M. Murry, C. Chen and A. Lee. 1979. Survey of the manufacture, import and uses for benzidine, related substances and related dyes and pigments, EPA Report 560/13-79-005. Environmental Protection Agency, Washington,D.C.

Prival, M.J., S.J. Bell, V.D. Mitchell, M.D.Peiperl and V.L. Vaughan. 1984. Mutagenicity of benzidine and benzidine-congener dyes and selected monoazo dyes in a modified salmonella assay. Mutat. Res. **136**:33-47.

Prival, M.J., V.M. Davis, M.D. Peiperl and S.J. Bell. 1988. Evaluation of azo food dyes for mutagenicity and inhibition of mutagenicity by methods using *Salmonella typhimurium.* Mutat. Res. **206**:247-259.

Prival, M.J. and V.D. Mitchell. 1982. Analysis of a method for testing azo dyes for mutagenic activity in *Salmonella typhimurium* in the presence of flavin mononucleotide and hamster liver S9. Mutat. Res. **97**:103-116.

Purchase, I.F.H. 1994. Current knowledge of mechanisms of carcinogenicity: Genotoxins versus non-genotoxins. Human Exper. Toxic. **13**:17-28.

Radman, M. 1976. An endonuclease from *Escherichia coli* that introduces single polynucleotide chain scissions in ultraviolet-irradiated DNA. J. Biol. Chem. **251**:1438-1734.

Radomski, J.L. 1961. The absorption, fate and excretion of citrus red no. 2 (2,5-dimethoxyphenyl-azo-2-naphthol) and ext. D and C red No. 14 (1-xylylazo-2-naphthol). J. Pharmacol. Exp. Ther. **134**:100-109.

Rafii, F. and C.E. Cerniglia. 1990. An anaerobic nondenaturing gel assay for the detection of azoreductases from anaerobic bacteria. J. Micro. Methods **12**:139-148.

Rafii, F. and C.E. Cerniglia. 1993. Localisation of the azoreductase of *Clostridium perfringens* by immuno-electron microscopy. Curr. Microbiol. **27**:143-145.

Rafii, F., W. Franklin and C.E. Cerniglia. 1990. Azoreductase activity of anaerobic bacteria isolated from human intestinal microflora. Appl. Environ. Microbiol. **56**:2146.

Rafii F., D.B. Smith, R.W. Benson and C.E. Cerniglia. 1992. Immunological homology among azoreductases from *Clostridium* and *Eubacterium* strains isolated from human intestinal microflora. J. Basic Microbiol. **32**:99-105.

Rahimtula, A.D., B. Jernstrom, L. Dock and P. Moldeus. 1982. Effects of dietary and *in vitro* 2<3>-t-butyl-4-hydroxy-anisole and other phenols on hepatic enzyme activities in mice. Br. J. Cancer **45**:935-944.

Ramotar, D., S.C. Popoff and B. Demple. 1991. Complementation of DNA repair-deficient *Escherichia coli* by yeatst Apn1 apurinic/apyrimidinic endonuclease gene. Mol. Bicrobiol. **5**:149-155

Ramotar, D., S.C. Popoff, E.B. Gralla and B. Demple. 1991a. Cellular role of yeast Apn1 apurinic endonuclease/3'-diesterase: repair of oxidative and alkylation DNA damage and control of spontaneous mutation. Mol. Cell Biol. **11**:4537-4544.

Rao, M.S. and J.K. Reddy. 1987. Peroxisome proliferation and hepatocarcinogenesis Carcinogenesis **8**:361-366.

Reid, T.M., K.C. Morton, C.Y. Wang, and C.M. King. 1984. Mutagenicity of azo dyes following metabolism by different reductive/oxidative systems. Environ. Mutagen. **6**:705-717.

Richter C. 1987. Biophysical consequences of lipid peroxidation in membranes. Chem. Phys. Lipids **44**:175.

Richter, C. and B. Frei. 1988. Calcium release from mitochrondria induced by proxidants. Free Radical Biol. Med. **4**:365.

Richter, C., J.W. Park and B.N. Ames. 1988. Normal oxidative damage to mitochondrial and nuclear DNA is extensive. Proc. Natl. Acad. Sci. USA. **85**:6465-6467

Roe, F.J. 1989. Non-genotoxic carcinogenesis: implications for testing and extrapolation to man. Mutagenesis **4**:407-411.

Rosenkranz, H.S. and G. Klopman. 1990. Structural basis of the mutagenicity of 1-amino-2-naphthol-based azo dyes. Mutagenesis **5:**137-146.

Rosenkranz, H.S. and Z. Leifer. 1980. Determining the DNA modifying activity of chemicals using DNA-polymerase-deficient *Escherichia coli,* in:Chemical Mutagens, principles and methods for their detection. Vol. 6, de Serres, F.J. and A. Hollaender (eds.) Plenum Press, New York and London, pp 109-147.

Rossi, M.A., F. Fidale, A. Garramone, H. Esterbauer and M.U. Dianzani. 1990. Effect of 4-hydroxyalkenals on hepatic phosphatidylinositol-4,5-bisphosphate-phospholipase C. Biochem. Pharmacol. **39**:1715-1719.

Rowland, I.R. 1988. Factors affecting metabolic activity of the intestinal microflora. Drug Metab. Rev. **19**:243-261.

Rowland, I.R, A. Wise and A.K. Mallet. 1983. Metabolic profile of caecal microorganisms from rats fed indigestible plant cell-wall components. Food Chem. Toxicol. **21**:25.

Roxon, J.J., A.J. Ryan and S.E. Wright. 1966. Reduction of tartrazine by a *Proteus* species isolated from rats. Food Cosmet. Toxicol. **4:**419.

Roxon, J.J., A.J. Ryan and S.E. Wright. 1967. Reduction of water soluble azo dyes by intestinal bacteria. Food Cosmet. Toxicol. **5**:367-369.

Ryan, A.J. and S.E. Wright. 1961. The excretion of some azo dyes in rat bile. J. Pharm. Pharmacol. **13**:492-495.

Sakumi, K. and M. Sekiguchi. 1989. Regulation of expression of the ada gene controlling the adaptive reponse. Interactions with the ada promoter of the Ada protein and RNA polymerase. J. Mol. Biol. **205**:373-385.

Sancar, A. and Sancar, G.B. 1988. DNA repair enzymes. Annu. Rev. Biochem. **57**:29-67.

Sasaki, T. and T. Yoshida. 1935. Experimentelle erzeugung des lebercarcinoms durch futterung mit o-amidoazotoluol. Virchows Arch. Pathol. Anat. **295**:175-200.

Sawyer D.T. and J.S. Valentine. 1981. How super is superoxide? Acc. Chem. Res. **14**:393.

Scheline, R.R. and B. Longberg. 1965. The absorption, metabolism and excretion of the sulphonated azo dye, acid yellow, by rats. Acta. Pharmacol. Toxicol. **23:**1-14.

Scheline, R.R., R.T. Nygaard and B. Longberg. 1970. Enzymatic reduction of the azo dye, acid yellow, by extracts of *Streptococcus faecalis* isolated from rat intestine. Fd. Cosmet. Toxicol. **8**:55-58.

Schilderman, P.A.E.L., F.J. ten Vaarwerk, J.T. Lutgerink, A. Van der Wurff, F. ten Hoor and J.C.S. Kleinjans. 1995. Induction of oxidative DNA damage and early lesions in rat gastrointestinal epithelium in relation to prostaglandin H synthase-mediated metabolism of butlyated hydroxyanisole. Fd. Chem. Toxic. **33**:99-109.

Schmahl, D. and G. Eisenbrand. 1982. Influence of ascorbic acid on the endogenous (intragastral) formation of N-nitroso compounds. Int. J. Vitam. Nutr. Res. Suppl. **23**:91-102.

Schmahl, D., R. Preussmann, R. and M.R. Berger. 1989. Causes of cancer - an alternative view to Doll and Peto (1981). Klin Wochenschr **67**:1169-1173.

Schneider, W. and H. Staudinger. 1965. Reduced nicotinamide-adenine dinucleotide-dependent reduction of semidehydroascorbic acid. Biochim. Biophys. Acta. **96**:157-162.

Schraufstatter, I.U., P.A. Hyslop, J. Jackson and C.G. Cochrane. 1988. Cellular targets of hydrogen peroxide and hydrochlorous acid induced injury. Basic Life Sci. **49**:833-837.

Schwarz, L.R. and H. Greim. 1986. Environmental chemicals in hepatocarcinogenesis: the mechanism of tumor promotors. Prog. Liver Dis. **8**:581-595.

Segerback, D. 1983. Alkyation of DNA and haemoglobin in the mouse following exposure to ethane and ethane oxide. Chem. Biol. Interact. **45**:135-151.

Seis, H. and C.F.M. Menck. 1992. Singlet oxygen induced DNA damage. Mutat. Res. **275**:367-375.

Shacter E., R.L. Lopez, E.J. Beecham, S.Janz. 1990. DNA damage induced by phorbol ester-stimulated neutrophils is augmented by extracellular cofactors. Role of histidine and metals. J. Biol. Chem. **265**:6693-6699.

Shahin, M.M. 1987. Relationships between structure and mutagenic activity of environmental chemicals. Mutat. Res. **181**:243-256.

Shahin M.M. 1989. Evaluation of the mutagenicity of azo dyes in *Salmonella typhimurium:* a study of structure-activity relationships. Mutagenesis **4**:115-125.

Shargel, L., A.R. Banijamali and S.H.Kuttab. 1984. Relationship between azo dye structure and rat hepatic azoreductase activity. J. Pharm. Sciences **73:**161-164.

Sharma, R.C. and K.C. Smith. 1985. A minor pathway of postreplication repair in *Escherichia coli* is independent of the recB, recC and recF genes. Mutat. Res. **146**:169-176.

Shibutani, S., M. Takeshita and A.P. Grollman. 1991. Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. Nature **349**:431-434.

Shigenaga, M.K., T.M. Hagen and B.N. Ames. 1994. Oxidative damage and mitochondrial decay in ageing. Proc. Natl. Acad. Sci. USA. **91**:10771-10778.

Shimizu, H., Y. Suzuki, N. Takemura, S. Goto and H. Matsushita. 1985. The results of microbial mutation test for forty-three industrial chemicals. Jpn. J. Ind. Hlth. **27**:400-419.

Shklar, G., J. Schwartz, D. Trickler and S. R. Cheveries. 1993. The effectiveness of a mixture of β-carotene, α-tocoperol, glutathione and ascorbic acid for cancer prevention. Nutr. Cancer **20**:145-151.

Singh, A. 1982. Chemical and biochemical aspects of superoxide radicals and related species of activated oxygen. Can. J. Physiol. Pharmacol. **60**:1330-1345.

Slater. E.E., M.D. Anderson and H.S. Rosenkranz. 1971. Rapid detection of mutagens and carcinogens. Cancer Res. **31**:970-973.

Smith, K.C. and D.H.C. Meun. 1970. Repair of radiation induced damage in *Escherichia coli.* II. Effect of *rec* mutation on postreplication repair of damage due to ultraviolet radiation. J. Molec. Biol. **51**:459-472

So. K.O., P.K. Wong and K-Y. Chan. 1990. Decolorization and biodegradation of methyl red by *Acetobacter liquefaciens.* Toxicity Assessment: An International Journal **5**:221-235.

Soderland, E.J., E. Dybing, S. Nordenson and E. Tjelta. 1980. The role of ethyl and gluorine substitution in the 4' position for *N,N*-dimethyl-4-aminoazobenzene mutagenicity and azo reduction, Acta Pharmacol. Toxicol. **47**:175-182.

Spadaro, J.T., M.H. Gold and V. Renganathan. 1992. Degradation of azo dyes by the lignin-degrading fungus *Phanerochaete chrysosporium.* Appl. Environ. Microbiol. **58**:2397-2401.

Spitz, S., W.H. Maguinan and K. Dobriner. 1950. Carcinogenic action of benzidine. Cancer **3**:789-804.

Stadtman, E.R. and C.N. Oliver. 1991. Metal-catalysed oxidation of proteins. Physiological consequences. J. Biol. Chem. **266**:2005-2008.

Stead, C.V. 1970. Azo dyes, *In* The Chemistry of Synthetic Dyes, Vol 3, (K. Venkataraman, ed.) Academic Press, New York, Chap. 6.

Steenken, S. 1989. Structure, acid/base properties and transformation reactions of purine radicals. Chem. Rev. **89**:503-520

Stevens, R.G., D.Y. Jones, M.S. Micozzi and P.R. Taylor. 1988. Body iron stores an the risk of cancer. New Engl. J. Med. B319:1047-1052.

Stiborova, M., B. Asfaw, P. Anzenbacher and P. Hodek. 1988a. A new way to carcinogenicity of azo dyes: The benzenediazonium ion formed from a non-aminoazo dye, 1-phenyl-2-hydroxy-naphthalene (sudan I) by microsomal enzymes binds to deoxyguanosine residues of DNA. Cancer Letts. **40**:327-333.

Stiborova, M., B. Asfaw, P. Anzenbacher, L. Leseticky and P. Hodek. 1988b. The first identification of the benzenediazonium ion formation from a non-aminoazo dye, 1-phenyl-2-hydroxy-naphthalene (sudan I) by microsomes of rat livers. Cancer Letts. **40**:319-326.

Stier, A., R. Clauss, A. Lucke and I. Reitz. 1980. Redox cycle of stable mixed nitroxides formed from arcinogenic aromatic amines. Xenobiotica **10**:661-673.

Storz, G., M.F. Christman, H. Sies and B.N. Ames. 1989. Spontaneous mutagenesis and oxidative damage to DNA in *Salmonella typhimurium.* Proc. Natl. Acad. Sci. USA **84**:8917-8921.

Storz, G., L.A. Tartaglia, S.B. Farr and B.N. Ames. 1987. Bacterial defenses against oxidative stress. Trends Genet. **6**:363-368.

Sulivan , N., D. Gatehouse and D. Tweats. 1993. Mutation, cancer and transgenic models: relevance to the toxicology industry. Mutagenesis **8:**167-174.

Summerfield, F.W. and A.L. Tappel. 1983. Determination by fluorescence quenching of the environment of DNA crosslinks made by malondialdehyde. Biochem. Biophys. Acta. **740**: 185-189.

Tagesson, C., D. Chabiuk, O. Axelson, B. Baranski, J. Palus and K. Wyszynska. 1993. Increased urinary excretion of the oxidative DNA adduct, 8-hydroxydeoxyguanosine, as a possible early indicator of occupational cancer hazards in the asbestos, rubber and azo-dye industries. Pol. J. Occ. Med. Environ. Health **6**:357-368.

Tagesson, C., M. Kallberg and P. Leandersson. 1991. Determination of urinary 8-hydroxydeoxyguanosine by coupled column HPLC with electrocchemical detection: A noninvasive assay for *in vivo* oxidative DNA damage in humans. Toxicol. Methods **4**:242-251.

Tanaka, K., S. Marui and T. Mii. 1980. Mutagenicity of extracts of urine from rats treated with aromatic amines. Mutat. Res. **79**:173-176.

Tchou, J. and A.P. Grollman. 1993. Repair of DNA containing the oxidatively damaged base, 8-oxoguanine. Mutat. Res. **299**:277-287.

Tchou, J., H. Kasai, S. Shibutani, M.H. Chung, J. Laval, A.P. Grollman and S. Nishimura. 1991. 8-Oxoguanine (8-hydroxyguanine) DNA glycosylase and its substrate specificity. Proc. Natl. Acad. Sci. USA **88**:4690-4694.

Tennant, R.W. and J. Ashby. 1991. Classification according to chemical structure, mutagenicity to Salmonella and level of carcinogenicity of a further 39 chemicals tested for carcinogenicity by the US National Toxicology Programme, Mutat. Res. **257**:209-228.

Testa B. and P. Jenner. 1976. Drug metabolism: chemical and biological aspects. Marcel Dekker, New York.

Thliveris, A.T., J.W. Little and D.W. Mount. 1991. Repression of the *Escherichia coli* recA gene requires at least two LexA protein monomers. Biochimie. **73**:449-456.

Thompson, L.H. 1989. Somatic cell genetics approach to dissecting mammalian DNA repair. Environ. and Mol. Mutagenesis. **14**:264-281.

Thorstensen, K. and I. Romslo. 1990. The role of transferrin in the mechanism of cellular iron uptake. Biochem. J. **271**:1-9.

Tsaneva, I.R., B. Muller and S.C. West. 1992. ATP-dependent branch migration of Holliday junctions promoted by the RuvA and RuvB proteins of *Escherichia coli*. Cell **69**:1171-1180.

Tsaneva, I.R. and B. Weiss. 1990. *soxR*, a locus governing a superoxide response regulon in *Escherichia coli* K-12. J. Bacteriol. **172**:4197-4205.

Tweats, D., M. Green and W.J. Muriel. 1981. A differential kill assay for mutagens and carcinogens based on an approved repair deficient strain of *Escherichia coli*. Carcinogenesis **2**:189-195.

Ursini, F., M. Maiorino and C. Gregolin. 1985. The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. Biochim. Biophys. Acta **839**:62.

van Acker, S.A.B.E., L.M.H. Koymans and A. Bast. 1993. Molecular pharmacology of vitamin E: structural aspects of antioxidant activity. Free Radical Biol. Med. **15**:311-328.

van Kuijk F.J., G.M. Handelman and E.A. Dratz. 1985. Consecutive action of phospholipase A2 and glutathione peroxidase is required for reduction of phospholipid hydroperoxides and provides a convenient method to determine peroxide values in membranes. J. Free Radic. Biol. Med. **1**:421-427.

Vance, W.A., H.S. Okamoto and Y.Y. Wang. 1986. Structure-activity relationships of nitro and methyl-nitro derivatives of indoline, indole, indazole and benzimidazole in *Salmonella typhimurium*. Mutat. Res. **173**:169-176.

Venitt, S. and C.T. Bushell. 1976. Mutagenicity of the food colour brown FK and constituents in *Salmonella typhimurium*. Mutat. Res. **40**:309-316.

Venitt, S., R. Forster and E. Longstaff. 1983. Bacterial Mutation Assays, in: UKEMS Subcommittee on Guidelines for Mutagenicty Testing. Report, Part I. Basic Test Battery, B.J. Dean (ed.) UK Environmental Mutagen Society, Swansea.

Venkataraman, K. 1952. The chemistry of synthetic dyes. Academic Press. New York.

von Sonntag, C. 1987. The chemical basis of radiation biology. Taylor and Francis, London.

Walker, R. 1970. The metabolism of azo compounds, a review of the literature. Food Cosmet. Toxicol. **8**:659-679.

Walker, R. 1973. The influence of gut micro-organisms on the metabolsm of drugs and food additives. Proc. Nutr. Soc. **32**:73-77.

Walker, R., R. Gingell and D.F. Murrells. 1971. Mechanisms of azo reduction by *Streptococcus faecalis* I. Optimization of assay conditions. Xenobiotica **1**:221-229.

Walker R., and A.J. Ryan. 1971. Some molecular parameters influencing rate of reduction of azo compounds by intestinal microflora. Xenobiotica **1**:483-486.

Wallace, S.S. 1988. AP endonucleases and DNA gylcosylases that recognise oxidative DNA damage. Environ. Mol. Mutagen. **12**:431-477.

Walters, J.M. and R.D. Combes. 1983. Evaluation of a methodology for the use of preparations from rat small intestine in the Salmonella/microsome assay. Mutat. Res. **113**:393-402.

Ward, J.F., C.L. Limoli, P. Calabro-Jones and J.W. Evans. 1987. Radiation vs chemical damage to DNA. In Cerutti P.A *et al.* (eds.): Anticarcinogenesis and radiation protection. New York, Plenum Press, pp 321-327.

Watabe T., N. Ozawa and F. Kobayashi. 1980. Reduction of sulphonated water-soluble azo dyes by micro-organisms from human faeces. Food Cosmet. Toxicol. **18**:349-352.

Weinstein, J. and B.H.J Bielski. 1979. Kinetics of the interaction of hydroxyl and superoxide radicals with hydrogen peroxide. The Haber-Weiss reaction. J. Am. Chem. Soc. **101**:58-62

Weisburger, E. K. 1978. Mechanisms of chemical carcinogenesis. Ann. Rev. Pharmacol. Toxicol. **18**:395-415.

Weisburger E.K. 1983. Species differences in response to aromatic amines. Basic Life Sci. **24**:23-47.

Weisburger E.K. and E.S. Fiala. 1981. Mechanisms of species, strain and dose effects in arylamine carcinogenesis. Natl. Cancer Inst. Monogr. **58**:41-48.

Weisburger E.K. and G.M. Williams. 1981. Carcinogen testing. Current problems and new approaches. Science **214**:401-407.

West, S.C. 1992. Enzymes and molecular mechanisms of genetic recombination. Annu. Rev. Biochem. **61**:603-640.

Wilcox, P., A. Naidoo, D.J. Wedd and D.G. Gatehouse. 1990. Comparison of *Salmonell typhimurium* TA102 with *Escherichia coli* WP2 tester strains. Mutagenesis **5**:285-291.

Wills, E.D. 1971. Effect of lipid peroxidation on membrane bound enzymes of the endoplasmic reticulum. Biochem. J. **37**:403.

Winquist, L, U. Rannug, A. Rannug and C. Ramel. 1984. Protection from toxic and mutagenic effects of hydrogen peroxide by catalase induction in *Salmonella typhimurium.* Mutat Res. **141**:145-147.

Winterbourn, C.C and H.C. Sutton. 1986. Iron and xanthine oxidase catalyse formation of an oxidant species distinguishable from hydroxyl radical: comparisons with the Haber-Weiss reaction. Arch. Biochem. Biophys. **244**:27.

Wise, A., A.K. Mallett and I.R. Rowland. 1983. Dietary protein and caecal microbial metabolism in the rat. Nutr. Cancer **4**:267.

Witkin, E.M. 1976. Ultraviolet mutagenesis an inducible DNA repair in *Escherichia coli.* Bact. Rev. **40**:869-907.

Wood, M.L., M. Dizdaroglu, E. Gajewski and J.M. Essigmann. 1990. Mechanistic studies of ionising radiation and oxidative mutagenesis: genetic effects of a single 8-hydroxyguanine (7-hydro-8-oxoguanine) residue inserted at a unique site in a viral genome. Biochem. **29**:7024-7032.

Wuhrmann, K., K. Mechsner and T. Kappeler. 1980. Investigation on rate - determining factors in the microbial reduction of azo dyes. European J. Appl. Microbiol. **9**:325-338.

Wyrobek, A.J., L.A. Gordon, J.G. Burkhart, M.W. Francis, R.W. Kapp, G. Letz, H.V. Malling, J.C. Topham and M.D. Whorton. 1983. An evaluation of human sperm as indicators of chemically induced alterations of spermatogenic function. A report of the US Environmental Protection Agency Gene-Tox Program. Mutat. Res. **115**:73-148.

Yahagi, T., M. Degawa, Y. Seino, T. Matsushima, M. Nagao, T. Sugimura and Y. Hashimoto. 1975. Mutagenicity of carcinogenic azo dyes and their derivatives. Cancer Letts., **1**:91-96.

Yahagi, T., M. Nagao, Y. Seino, T. Matsushima, T. Sugimura and M. Okada. 1977. Mutagenicities of N-nitrosamines on Salmonella. Mutat. Res. **48**:121-130.

Yatome, C., T. Ogawa, H. Hayashi and T. Ogawa. 1991. Microbial reduction of azo dyes by several strains. J Environ. Sci. Health **A26**:471-485.

Yatome, C., T. Ogawa, H. Hishida and T. Taguchi. 1990. Degradation of azo dyes by cell free extract from *Pseudomonas stutzeri.* JSDC **106**:280-283.

Yatome, C., T. Ogawa, K. Itoh, A. Sugiyama and E. Idaka. 1987. Degradation of azo dyes by cell free extract from *Aeromanas hydrophila* var. 24B. JSDC **103**:395-398.

Yoshimura, Y., Y. Matsuzaki, K. Uchiyama, T. Watanabe, K. Ohsawa and K. Imaeda. 1993. Formation of 8-hydroxydeoxyguanosine from deoxyguanosine by Fe^{2+}/Ascorbic acid/EDTA/H_2O_2 system. J. Clin. Biochem. Nutr. **15**:155-162.

Zbaida, S., C.F. Brewer and W.G. Levine. 1994. Hepatic microsomal azoreductae activity: Reactivity of azo dye substrates is determined by their electronic densities and redox potentials. Drug Metabolism and Disposition **22**:412-418.

Zbaida, S. and W.G.Levine. 1990. Characteristics of two clases of azo dye reductase activity associated with rat liver microsomal cytochrome P450. Biochem. Pharm. **40:**2415-2423.

Zbaida, S. and W.G. Levine. 1991. A novel application of cyclic voltammetry for direct investigation of metabolic intermediates in microsomal azo reduction. Chem. Res. Toxicol. **4**:82-88.

Zbaida, S. and W.G. Levine. 1992. Role of electronic factors in binding and reduction of azo dyes by hepatic microsomes. J. Pharmacol. Exp. Ther. **260**: 554-561.

Zhou W. and W. Zimmermann. 1993. Decolorisation of industrial effluents containing reactive dyes by actinomycetes. FEMS Microbiol. Letts. **107**:157-162.

Zeiger, E. 1993. Mutagenicity of chemicals added to foods. Mutat. Res. **290**:53-61.

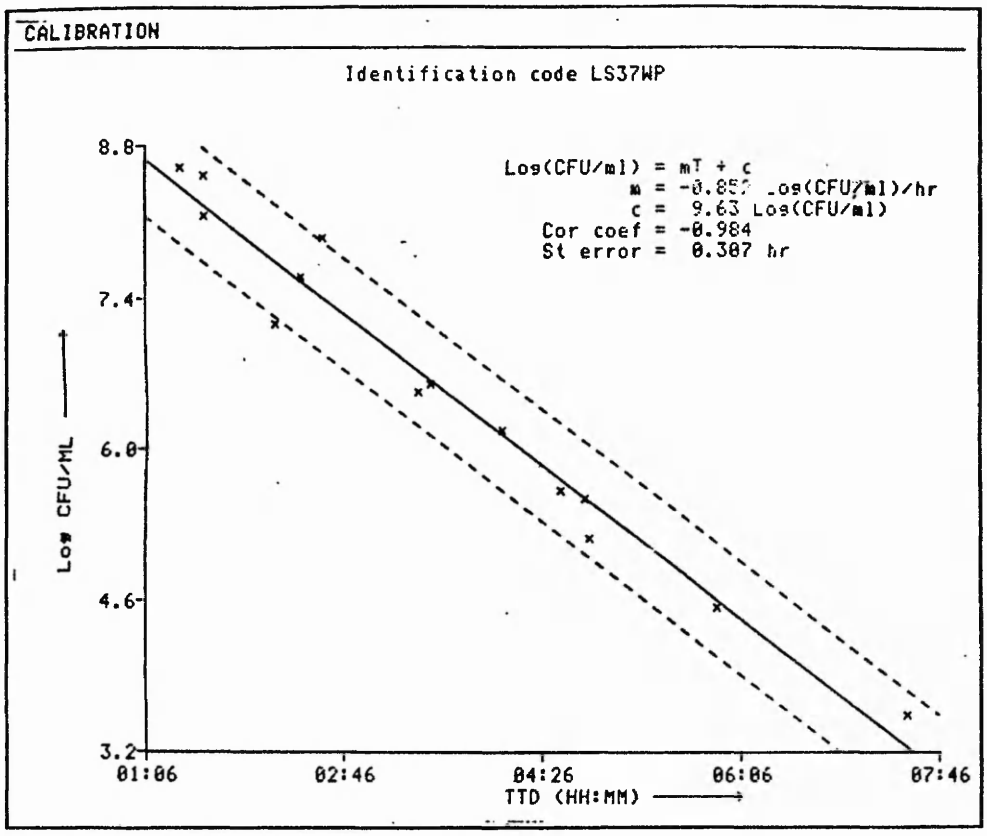
Zimmerman, T., H.G. Kulla and T. Leisenger. 1982. Properties of purified orange II azoreductase, the enzyme initiating azodye degradation by *Pseudomonas* KF46. Eur. J. Biochem. **129**:197-203.

APPENDIX 1.

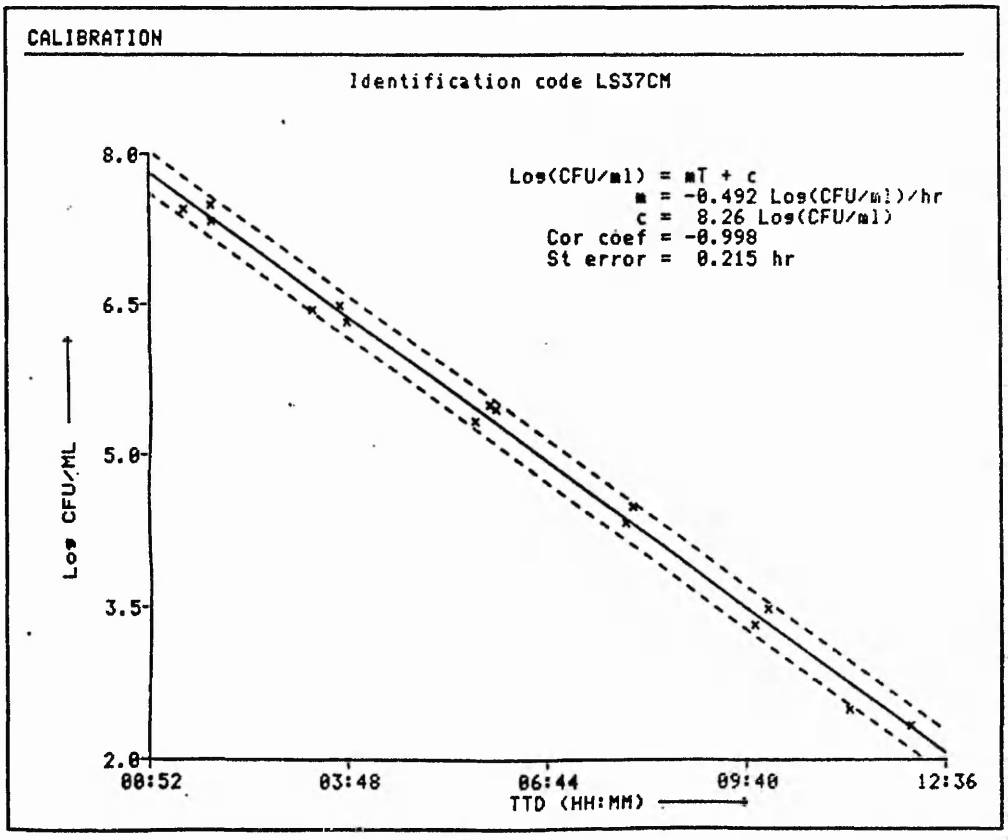
RABIT CALIBRATION CURVES

Appendix 1. RABIT calibration curves

E. coli WP2

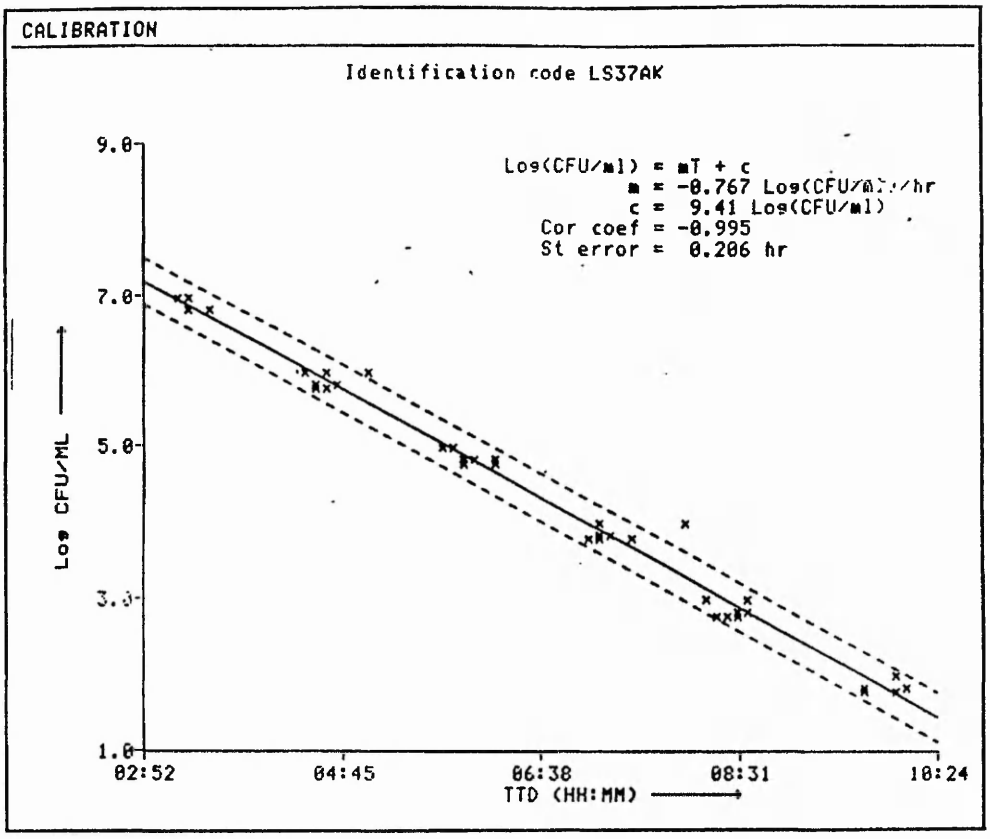


E. coli CM871

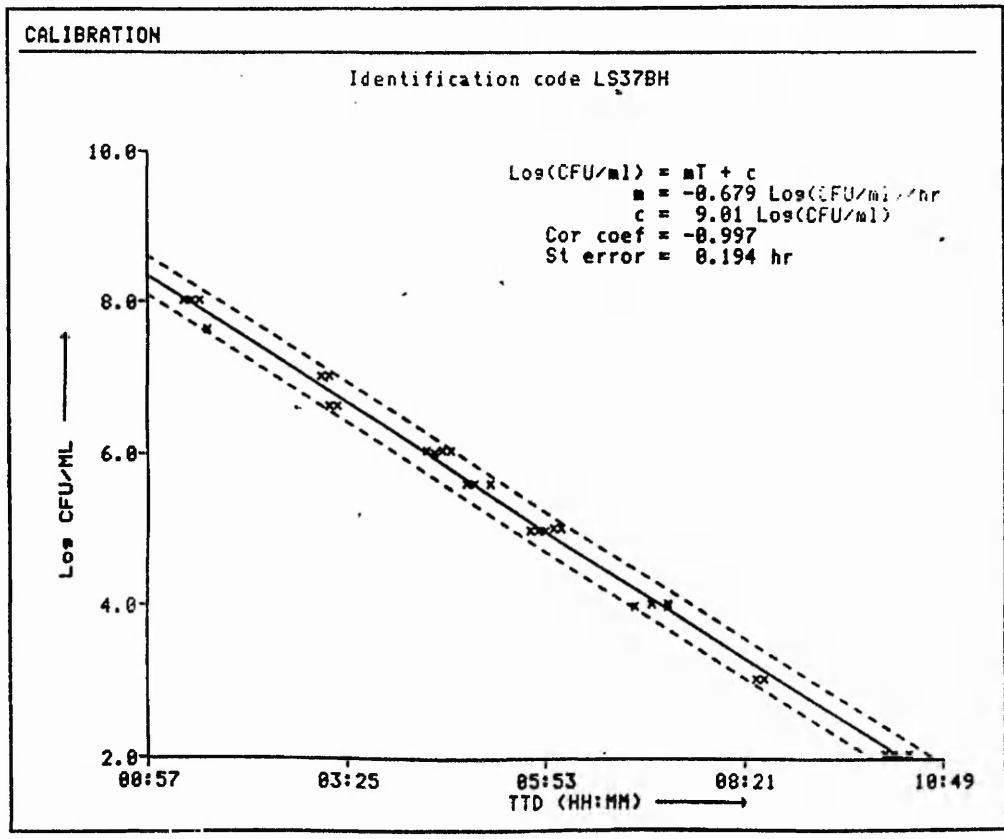


Appendix 1. RABIT calibration curves

E. coli AB1157



E. coli BH20



APPENDIX 2.

PUBLICATIONS

Evidence for Direct-acting Oxidative Genotoxicity by Reduction Products of Azo Dyes

Elizabeth A. Sweeney,[1] J. Kevin Chipman,[2] and Stephen J. Forsythe[1]

[1]Department of Life Sciences, The Nottingham Trent University, Nottingham, England; [2]School of Biochemistry, The University of Birmingham, Birmingham, England

The intestinal flora forms a complex ecosystem that metabolizes dietary and endogenous nutrients under primarily anaerobic conditions. The ingestion of azo dyes has been proposed as one source of potential genotoxic agents. Many intestinal bacteria are able to reduce the azo bond (termed azofission), which liberates the substituted naphthol compounds. The standard Ames test has not demonstrated mutagenicity either by various common food colorings or by their reduced end products in *Salmonella typhimurium* strains TA98 and TA100. In contrast, genetic toxicity was demonstrated in the *Escherichia coli* differential kill assay and in *S. typhimurium* TA102 for the reduced dyes. The superoxide free radical was produced by the azo dyes only after reduction by the intestinal bacteria *Enterococcus faecalis* and *Bacteroides thetaiotaomicron*. — Environ Health Perspect 102(Suppl 6):119–122 (1994)

Key words: azo dyes, *Salmonella typhimurium* TA102, superoxide radicals, azo reduction, oxidative mutagens, genotoxicity

Introduction

Azo compounds are the most common synthetic colorings used in the food, pharmaceutical, and cosmetic industry. Also known as coal tar dyes, they contain an aromatic ring linked by an azo bond to a second naphthalene or benzene ring. Coloring matter entering the intestinal tract is subjected to the action of acid, digestive enzymes, and microflora. Azo compounds may reach the intestine directly after oral ingestion or through the bile after parenteral administration. They are reduced by azo reductases from intestinal bacteria and, to a lesser extent, by enzymes of the cytosolic and microsomal fractions of the liver. The first catabolic step in the reduction of azo dyes, which is accompanied by a decrease in the visible light absorbance and then decoloration of the dye, is the reduction of the azo bond to produce aromatic amines (Figure 1). Aromatic amines, some of which are known carcinogens, have been found in the urine of dyestuff workers and test animals following administration of azo dyes (*1*).

Although a number of commonly used dyes are not mutagenic in *Salmonella typhimurium* strains TA98 and TA100 even after azoreduction, the production of reactive oxygen species from *o*-hydroxy aromatic amine products has been suggested (*2,3*). This article reports the generation of superoxide anions from reduced azo dyes and aminonaphthols and the genetic toxicity of these products in *Escherichia coli* and a *Salmonella* strain (TA102), which is sensitive to oxidants.

Materials and Methods

Bacterial Strains

The organisms studied were obtained from The Nottingham Trent University Culture Collection (England), except *S. typhimurium* strains, which were obtained from B. Ames, University of California, Berkeley.

Enterococcus faecalis was grown in tryptone soya broth, and *Bacteroides thetaiotaomicron* was grown anaerobically in Schaedlers broth; both were incubated at 37°C for 24 hr. Cells were harvested by centrifugation at 7890*g* for 10 min and anaerobically washed once with 0.5 M potassium phosphate buffer, pH 7.4. The bacterial pellets were resuspended to one-twentieth of their previous volume in phosphate buffer and immediately used for azo reduction.

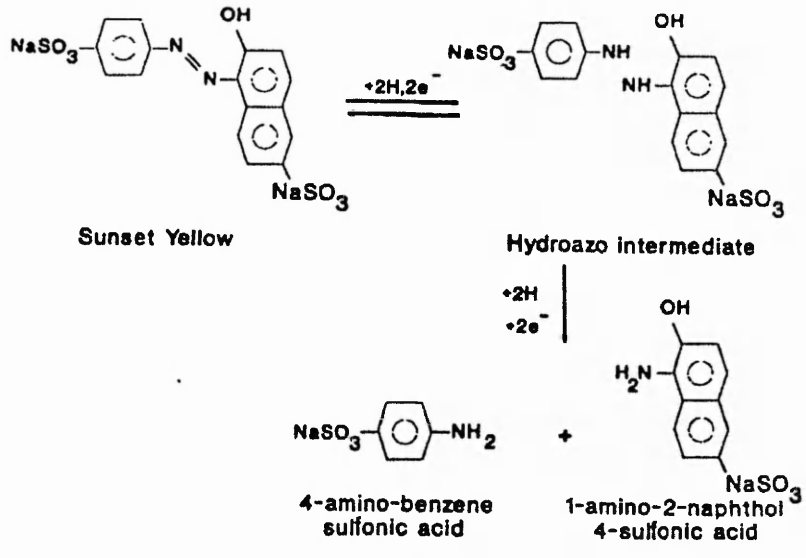


Figure 1. Possible reduction mechanism for sunset yellow.

This paper was presented at the Fifth International Conference on Carcinogenic and Mutagenic *N*-Substituted Aryl Compounds held 18–21 October 1992 in Würzburg, Germany.

E.A. Sweeney is grateful for financial support from The Nottingham Trent University.

Address correspondence to E. A. Sweeney, Department of Life Sciences, The Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, UK. Telephone 0602 418418. Fax 0602 486636

Materials

Sunset yellow (Colour Index [C.I.] 15985) and carmoisine (C.I. 14720) were obtained from Gurr, BDH Chemicals Ltd (Poole, Dorset, UK). Amaranth (C.I. 16185) and all other chemicals used were obtained from Sigma Chemical Co. (Poole, Dorset, UK). It should be noted that purity of the azo dyes was 85%. The electron carriers used were flavin mononucleotide (FMN), riboflavin (RF), and flavin adenine dinucleotide (FAD).

Bacterial Reduction of Azo Dyes

The standard assay mix consisted of 4.2 ml degassed potassium phosphate buffer (50 mM, pH 7.4), 0.4 ml washed cell suspension with or without glucose (0.2 ml, 10% wt/vol) and electron carriers in narrow assay tubes (18 × 142 mm). The various electron carriers were prepared anaerobically at 0.025 mM. The reaction mix was incubated at 37°C prior to starting the reaction by the addition of 0.1 ml of the appropriate azo dye (10 mM). Decoloration rate was determined spectrophotometrically at the wavelength of the maximal absorbance of the azo dye.

Chemical Reduction Assay

Dyes were reduced either with sodium dithionite according to the method of Zbaida and Levine (*4*), or with stannous chloride as described by Gasparic (*5*).

Detection of Superoxide Anion Radical

Superoxide anion (O_2^-) production was detected by the reduction of nitroblue tetrazolium (NBT) and its inhibition by superoxide dismutase (SOD), according to the method of Oberley et al. (*6*). The sample (100 µl) to be tested was added to 0.8 ml potassium phosphate buffer (50 mM, pH 7.8) containing 0.056 mM NBT, 0.1 mM EDTA, 0.1 mM xanthine, 0.06% wt/vol Triton X100, and 0.33 mg/ml gelatin, ± 100 µl SOD. Immediately after mixing, the absorbance at 560 nm was measured with respect to time. SOD activity was assayed using a xanthine/xanthine oxidase technique (*6*). SOD was inactivated by boiling for 15 min.

Differential Killing Assay

The *E. coli* differential cytotoxicity assay was used to determine the genotoxic activity of the azo dyes according to the method described by Tweats et al. (*7*). The tester strains were *E. coli* WP2, (repair proficient) and *E. coli* CM871 (repair deficient). *E. coli* CM871 is a uvrA recA lexA triple mutant that combines extreme repair deficiency with near wild-type growth. Compounds were accepted as genotoxic if survival of the repair deficient strain was at least 4-fold lower than survival of the repair proficient strain.

All compounds were tested for genotoxicity with and without prior incubation with either *E. faecalis* or *B. thetaiotaomicron* cell suspensions. Incubation was for 1 hr with *E. faecalis* or overnight with *B. thetaiotaomicron*. Final concentrations of agents used were azo dyes, 5 mM; 1-aminonaphthol HCl, 0.1 mM; 1-aminonaphthol-4-sulfonic acid, 10 mM; 4-aminonaphthalene HCl, 1 mM; and 4-aminonaphthalene-sulfonic acid sodium salt, 5 mM.

Genotoxic activity was assessed after removing cells by centrifugation in a microfuge (8000*g*, 5 min), followed by filtration (0.2 µm Dynaguard filters, New Brunswick). An aliquot (100 µl) of the filtrate was added to the diluted *E. coli* tester strain (200 µl), and the genotoxic activity was determined. Inhibition by UV was used as a positive control for CM871.

The Rapid Automated Bacterial Impedance Technique (RABIT), (Don Whitley Scientific Ltd, Shipley, UK) and plate counts were used to measure microbial survival (*8*).

Salmonella Mutagenicity Test

The protocol used was essentially that of Ames et al. (*9*). The strains of *S. typhimurium* used were TA98, TA100, and TA102. Liver postmitochondrial supernatant was not incorporated in this test. Bleomycin was used as a positive control for TA102 (*9*).

Results

The reduction of the three dyes by *E. faecalis* and *B. thetaiotaomicron* is summarized in Table 1. Azo reductase activity was stimulated by the addition of extracellular electron acceptors such as FMN, FAD, and RF. The presence of glucose on reduction of azo dyes was inhibitory for *B. thetaiotaomicron*, but not for *E. faecalis*. This may be because of the different electron transport systems of these organisms.

After incubation with *E. faecalis*, the cleavage products of sunset yellow and amaranth were found to be genotoxic in the bacterial differential assay (Figure 2). Carmoisine was marginally genotoxic after azo reduction, but survival of *E. coli* CM871 was not consistently 4-fold less than survival of *E. coli* WP2. Similar results were obtained on incubation with *B. thetaiotaomicron*. Unreduced dyes were not genotoxic in this assay. Of the four coupling components tested, 1-amino-2-naphthol HCl and 1-amino-2-naphthol-4-sulfonate are considered genotoxic.

Mutagenicity studies on the same samples using the standard Ames test with *S. typhimurium* TA98 and TA100 showed no mutagenicity. When compounds were retested using *S. typhimurium* TA102, reduced amaranth and reduced sunset yellow gave a doubling of the spontaneous reversion rate (Figure 3). This strain detects a variety of oxidants as mutagens that are not detected by strains TA98 or TA100 (*10*).

Generation of O_2^- was observed for reduced dyes and some aminonaphthols but not for unreduced dyes (Figures 4,5). The increase in absorbance at 540 nm (NBT assay) was inhibited by SOD, while inactivated SOD showed no inhibition. Table 2 summarizes the rates of O_2^- production from unreduced and reduced azo dyes and their coupling components. Results were further confirmed by an ability to reduce cytochrome *c* (data not shown). Negligible

Table 1. Cofactor requirements for azoreductase activity.[a]

Compound	Cofactor	Rate of decoloration, µmole/min/mg cell dry wt	
		by *E. faecalis*[b]	by *B. thetaiotaomicron*[c]
Amaranth	–	NQ	NQ
Amaranth	Gl	40.7 ± 2.4	NQ
Amaranth	FMN	NQ	7.2 ± 1.24
Amaranth	Gl + FMN	113.5 ± 7.04	3.9 ± 0.69
Amaranth	Gl + FAD	83.6 ± 3.5	5.5 ± 0.36
Amaranth	Gl + RF	75.6 ± 5.36	4.6 ± 0.17
Sunset yellow	–	NQ	NQ
Sunset yellow	Gl	79.0 ± 1.5	NQ
Sunset yellow	FMN	3.9 ± 0.17	7.5 ± 0.21
Sunset yellow	Gl + FMN	132.5 ± 4.11	5.41 ± 0.25
Sunset yellow	Gl + FAD	93.5 ± 1.98	6.3 ± 0.25
Sunset yellow	Gl + RF	78.1 ± 3.6	4.6 ± 0.48

Abbreviations: NQ, nonquantifiable (rate less than 1 nm dye reduced per minute per milligram cell dry weight); Gl, glucose; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide. [a]Mean ± SD of three independent exponents. [b]Cell dry weight = 0.76 mg/ml. [c]Cell dry weight = 0.51 mg/ml.

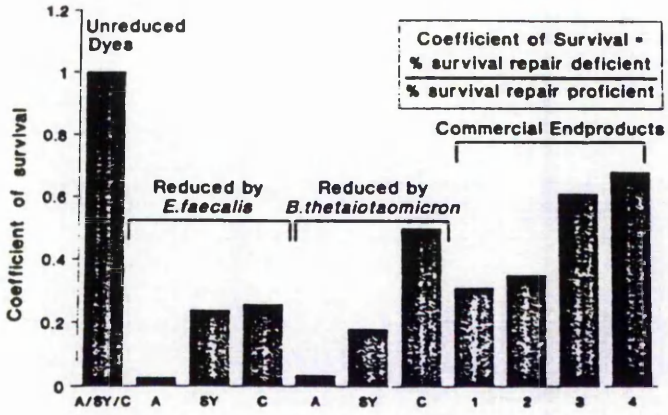


Figure 2. Bacterial genotoxicity of azo dyes using bacterial differential assay. A, amaranth; SY, sunset yellow; C, carmoisine; 1) 1-amino-2-naphthol hydrogen chloride; 2) 1-amino-2-naphthol-4-sulfonic acid; 3) 4-aminonaphthalene HCl; 4) 4-aminonaphthalene sulfonic acid, sodium salt.

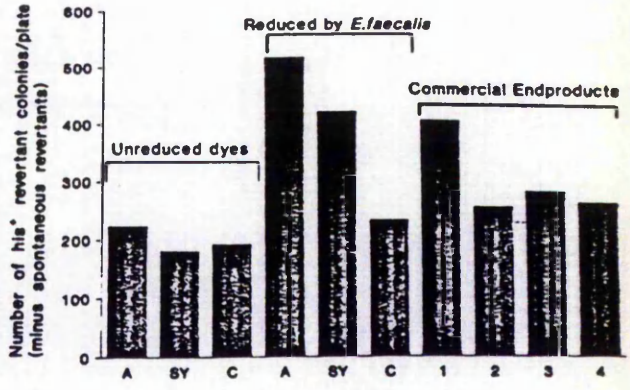


Figure 3. Bacterial mutagenicity of azo dyes, Ames test, and *S. typhimurium* TA102. A, Amaranth; SY, sunset yellow; C, carmoisine; 1) 1-amino-2-naphthol hydrogen chloride; 2) 1-amino-2-naphthol-4-sulfonic acid; 3) 4-aminonaphthalene HCl 4) 4-aminonaphthalene sulfonic acid, sodium salt. The mean number of viable bacteria and plate for spontaneous and control (1 mg/ml) incubations were 140 and 1050.

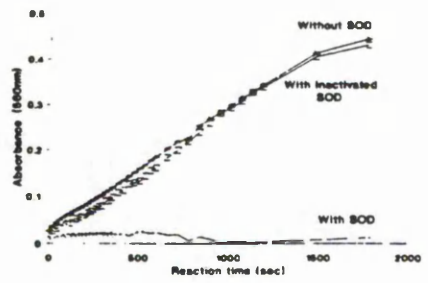


Figure 4. Superoxide production from sunset yellow reduced by *E. faecalis*.

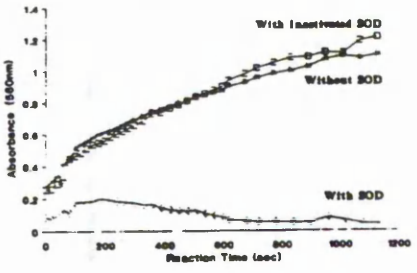


Figure 5. Superoxide production from sunset yellow reduced by stannous chloride.

O_2^- was produced by 4-aminonaphthalene and its sodium salt.

Discussion

It has been demonstrated that there are no cytochromes present in cell-free extracts of *E. faecalis* and that the transfer of electrons from reduced pyridine nucleotides to electron acceptors was mediated by a flavoprotein (*11*).

In view of their charged nature, it is uncertain whether FAD and FMN could readily shuttle across bacterial cell walls and membranes under physiologic conditions. In addition, it is unlikely that highly charged sulfonated dyes could pass through the cell wall of these organisms. An electron shuttle could affect the reduction with the dyes remaining extracellular. It may also serve as a partial explanation for the oxygen sensitivity of these reduction reactions, reduced flavin having a greater affinity for oxygen than it has for the azo dye.

In this article, we have tested three azo dyes for genotoxicity following bacterial reduction of the dye. Both amaranth and sunset yellow, when reduced, induced cytotoxicity indicating DNA damage in repair-deficient *E. coli* in the absence of hepatic enzymes, but they failed to mutate *S. typhimurium* strains TA98 and TA100. In contrast, strain TA102, which detects oxidative mutagens, (*10,12*) was mutated by reduced amaranth and reduced sunset yellow.

The findings suggest that various azo dye products are genotoxic, not through *N*-hydroxylation and esterification, which is characteristic of many aromatic amines (*2,13*), but rather through a mechanism involving oxygen radicals (*3,14*). In further support, the production of O_2^- radical from reduced azo dyes was detected by the

Table 2. Generation of O_2^- naphthalamines.[a]

Compound	Rate of O_2^- production, μmole compound/min
Xanthine	0.014 ± 0.0021
Amaranth	0
Carmoisine	0
Sunset yellow	0
Amaranth[b]	0.023 ± 0.0017
Carmoisine[b]	0.018 ± 0.0024
Sunset yellow[b]	0.026 ± 0.0011
Amaranth[c]	0.062 ± 0.0018
Carmoisine[c]	0.0198 ± 0.0034
Sunset yellow[c]	0.055 ± 0.0023
1-Amino-2-naphthol HCl	0.1065 ± 0.0045
4-Amino-2-naphthol sulfonic acid	0.022 ± 0.0026
4-Aminonaphthalene HCl	$1.336 \times 10^{-4} \pm 0.3 \times 10^{-5}$
4-Aminonaphthalene sulfonic acid, Na salt	$0.033 \times 10^{-4} \pm 0.4 \times 10^{-5}$

[a] Mean ± SD of three independent exponents. [b] Reduced by *E. faecalis*. [c] Reduced by $SnCl_2$.

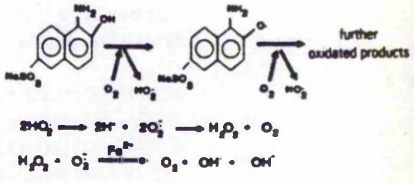


Figure 6. Possible mechanism for production of superoxide anions from reduced sunset yellow. Adapted from Nakayama (*3*).

reduction of nitrotetrazolium blue and confirmed using cytochrome *c*. The mechanism by which these species are formed is not clear, but they may be specific for *o*-hydroxy aromatic amines and involve the reactions postulated by Nakayama (*3*) and summarized in Figure 6. There may be a requirement for iron, as in Fenton chemistry, where O_2^- is converted via H_2O_2 to the highly reactive OH species that is known to damage DNA (*14*). All of the compounds shown to generate active oxygen species have a hydroxyl substituent *ortho* to the amino function. However, this does not explain the weakly mutagenic results for carmoisine. O_2^- was detected for 1-amino-2-naphthol HCl and 1-amino-2-naphthol sulfonate, while no active oxygen was detected for 4-aminonaphthalene HCl and its sodium salt.

We are currently studying the ability of *o*-hydroxy aromatic amines to produce deoxynucleotide oxidation in DNA. The identification of this type of DNA damage hitherto not detected by many conventional genotoxicity assays may have important implications regarding the continued use of azo dyes in foodstuffs.

REFERENCES

1. Cerniglia CE, Zhuo Z, Manning BW, Federle TW, Heflich RH. Mutagenic activation of the benzidine-based dye Direct Black 38 by human intestinal microflora. Mutat Res 175:11–16 (1986).
2. Kimura T, Kodama M, Nagata C. Nitroxide radicals generated from carcinogenic aminoazo dyes during their metabolism *in vivo* and in enzymatic systems *in vitro*. Biochem Pharmacol 28:557–560 (1979).
3. Nakayama T, Kimura T, Kodama M, Nagata C. Generation of hydrogen peroxide and superoxide anions from active metabolites of naphthalamines and aminoazo dyes: its possible role in carcinogenesis. Carcinogenesis 4:765–769 (1983).
4. Zbaida S, Levine WG. Role of electronic factors in binding and reduction of azo dyes by hepatic microsomes. J Pharmacol Exp Ther 260:554–561 (1992).
5. Gasparic J. Chemical degradation methods. In: The Analytical Chemistry of Synthetic Dyes (Venkataraman K, ed). New York:John Wiley and Sons, 1977;299–316.
6. Oberley LW, Spitz DR. Assay of superoxide dismutase activity in tumour tissue. In: Methods in Enzymology, Vol 105 (Packer L, ed). London:Academic Press, 1984;457–464.
7. Tweats D, Green M, Muriel WJ. A differential kill assay for mutagens and carcinogens based on an approved repair deficient strain of *Escherichia coli*. Carcinogenesis 2:189–195 (1981).
8. Forsythe SJ. The rapid detection of direct-acting DNA mutagens by electrical impedance with DNA repair deficient strains of *Escherichia coli*. Letts Appl Microbiol 11:30–32 (1990).
9. Ames BN, McCann J, Yamasaki E. Methods for detecting carcinogens and mutagens with *Salmonella*/mammalian microsome mutagenicity test. Mutat Res 31:347–364 (1975).
10. Levine D, Hollstein M, Christman M., Schwiers E, Ames BN. A new *Salmonella* tester strain (TA102) with A-T base pairs at the site of mutation detects oxidative mutagens. Proc Natl Acad Sci USA 79:7445–7449 (1982).
11. Gingell R, Walker R. Mechanisms of azo reduction by *Streptococcus faecalis* II. The role of soluble flavins. Xenobiotica 1:231–239 (1971).
12. DeFlora S, Camoirano A, Zanacchi C. Mutagenicity testing with TA97 and TA102 with 30 DNA damaging compounds, negative with other *Salmonella* strains. Mutat Res 134:159–165 (1984).
13. Farr SP, Kogoma T. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol Rev 55:561–585 (1991).
14. Basaga HS. Biochemical aspects of free radicals. Biochem Cell Biol 68:989–998 (1989).